IMPERIAL INSTITUTE
OF
AGRICULTURAL RESEARCH, PUSA.

PRINTED IN GREAT BRITAIN

CONTENTS OF VOLUME 35, Nos. 1 AND 2, 1941

(All rights reserved)

PAGE

1. Changes in phosphate distribution during anaerobic glycolysis in brain slices. By M. G. Macfarlane and H. Weil-Malherbe 1

2. Metabolism of normal and tumour tissue. 19. The metabolism of intestinal mucous membrane. By F. Dickens and H. Weil-Malherbe 7

3. The influence of dietary fibre on secretory activities of the alimentary tract. Observations on faecal phosphatase excretion and calcium and nitrogen balances of rats. By J. Duckworth and W. J. Godden 16

4. The component acids of phosphatides present in cow's milk fat. By T. P. Hilditch and L. Maddison 24

5. Palm oil carotenoids. 1. The isolation of lipoid pigments from a 'Sherbro' palm oil. By R. F. Hunter and A. D. Scott 31

6. The analysis of blood phospholipins. By W. N. M. Ramsay and C. P. Stewart. (With one figure) 39

7. The plasma-cell partition of blood lead. By S. L. Tompsett and A. B. Anderson 48

8. Studies in detoxication. 8. On the alleged formation of *p*-hydroxylaminobenzenesulphonamide and *p*-aminophenol from sulphanilamide *in vivo*, on the colour reactions used for their detection, and on the possible formation of aminophenolsulphonamides. By W. V. Thorpe, R. T. Williams and J. Shelswell . 52

9. Studies in detoxication. 9. The synthesis of some possible biological oxidation products of sulphanilamide. By W. V. Thorpe and R. T. Williams . . . 61

10. Production of amines by bacteria. 4. The decarboxylation of amino-acids by organisms of the groups *Clostridium* and *Proteus*. By E. F. Gale. (With seven figures.) With an addendum by G. L. Brown, F. C. MacIntosh and P. Bruce White 66

11. The colorimetric estimation of uric acid in fowl's excreta. By J. C. D. Hutchinson. (With one figure) 81

12. Separation of the higher monoamino acids by counter-current liquid-liquid extraction: the amino-acid composition of wool. By A. J. P. Martin and R. L. M. Synge. (With ten figures) 91

13. Enzymic proteolysis. 4. Amino-acids of casein phosphopeptone. By M. Damodaran and B. V. Ramachandran 122

14. The action of sulphites on the cystine disulphide linkages in wool. 2. The influence of temperature, time and concentration on the reaction. By F. F. Elsworth and H. Phillips. (With one figure) 135

15. A study of the isotopic constitution of potassium in various rat tissues. By A. Lasnitzki and A. K. Brewer. (With two figures) 144

16. The biochemistry of silicic acid. 9. Isolation and identification of minerals in lung residues and air-borne dusts from coal mines. By G. Nagelschmidt and E. J. King. (With Plate I) 152

17. Studies on diffusing factors. The hyaluronidase activity of testicular extracts, bacterial culture filtrates and other agents that increase tissue permeability. By D. McClean and C. W. Hale. (With seven figures) 159

[P.T.O.]

PAGE

18. Metabolism of polycyclic compounds. 4. Production of a dihydroxy-1:2:5:6-dibenzanthracene from 1:2:5:6-dibenzanthracene. By E. Boyland, A. A. Levi, E. H. Mawson and E. Roe. (With two figures) 184

19. Vitamin B_1 and bacterial oxidations. 1. Dependence of acetic acid oxidation on vitamin B_1. By J. H. Quastel and D. M. Webley. (With one figure) . . 192

20. The tryptophanase-indole reaction. 4. Some observations on the production of tryptophanase by *Esch. coli*; in particular the effect of the presence of glucose and amino acids on the formation of tryptophanase. By W. C. Evans, W. R. C. Handley and F. C. Happold. (With one figure) 207

21. Histidine metabolism in toxaemia of pregnancy. Isolation of histamine from the urine of patients with toxaemia of pregnancy. By R. Kapeller-Adler. (With one figure) 213

INDEX OF AUTHORS

PAGE

Anderson, A. B. 48

Boyland, E. 184
Brewer, A. K. 144
Brown, G. L. 79

Damodaran, M. 122
Dickens, F. 7
Duckworth, J. 16

Elsworth, F. F. 135
Evans, W. C. 207

Gale, E. F. 66
Godden, W. J. 16

Hale, C. W. 159
Handley, W. R. C. 207
Happold, F. C. 207
Hilditch, T. P. 24
Hunter, R. F 31
Hutchinson, J. C. D. 81

Kapeller-Adler, R. 213
King, E. J. 152

Lasnitzki, A. 144
Levi, A. A. 184

PAGE

McClean, D. 159
Macfarlane, M. G. 1
MacIntosh, F. C. 79
Maddison, L. 24
Martin, A. J. P. . . . 91
Mawson, E. H. 184

Nagelschmidt, G. 152

Phillips, H. 135

Quastel, J. H. 192

Ramachandran, B. V. 122
Ramsay, W. N. M. 39
Roe, E. 184

Scott, A. D. 31
Shelswell, J. 52
Stewart, C. P. 39
Synge, R. L. M. 91

Thorpe, W. V. 52, 61
Tompsett, S. L. 48

Webley, D. M. 192
Weil Malherbe, H. 1, 7
White, P. B. 79
Williams, R. T. 52, 61

CONTENTS OF VOLUME 35, No. 3, 194

(All rights reserved)

PAGE

22. Effect of diphtheria toxin upon tissue enzymes *in vitro*. By B. A. Peters and R. N. Cunningham 219

23. The system dehydroascorbic acid-glutathione. By E. M. Crook. (With seven figures) 226

24. A new method for the determination of phytohormone activity. By G. E. Turfitt. (With two figures) 237

25. The conversion of laevulose and fatty acid into glucose. By C. P. Stewart and J. C. Thompson 245

26. Observations on the serum phosphatase of cattle and sheep. By W. M. Allcroft and S. J. Folley 254

27. The determination of the viscosity of human milks and the prenatal secretions. By G. W. Scott Blair. (With two figures) 267

28. The viscosity, protein distribution, and 'gold number' of the antenatal and postnatal secretions of the human mammary gland. By H. Waller, R. Aschaffenburg and M. W. Grant 272

29. The colorimetric estimation of total cholesterol in whole blood, serum, plasma and other biological material. By J. T. Ireland. (With two figures) 283

30. Some applications of periodic acid to the study of the hydroxyamino-acids of protein hydrolysates. 1. The liberation of acetaldehyde and higher aldehydes by periodic acid. 2. Detection and isolation of formaldehyde liberated by periodic acid. 3. Ammonia split from hydroxyamino-acids by periodic acid. 4. The hydroxyamino-acid fraction of wool. 5. 'Hydroxylysine.' By A. J. P. Martin, R. L. M. Synge and F. O. Bell. (With Plate 2 and one figure) 294

31. Analysis of proteins. 13. Caseo-phosphopeptone. By J. Lowndes, T. J. R. Macara and R. H. A. Plimmer 315

32. Note on the amino-acids present in phosphopeptone. By C. Rimington . 321

33. Coupled oxidation of ascorbic acid and haemoglobin. 2. Formation and properties of choleglobin. By R. Lemberg, J. W. Legge and W. H. Lockwood. (With five figures) 328

34. Coupled oxidation of ascorbic acid and haemoglobin. 3. Quantitative studies on choleglobin formation. Estimation of haemoglobin and ascorbic acid oxidations. By R. Lemberg, J. W. Legge and W. H. Lockwood. (With one figure) . . . 339

35. Coupled oxidation of ascorbic acid and haemoglobin. 4. The 'labile iron' in blood and its increase in choleglobin formation. By J. W. Legge and R. Lemberg . 353

36. Coupled oxidation of ascorbic acid and haemoglobin. 5. Studies on the formation of bile pigments from choleglobin and verdohaemochromogen and on their isolation from erythrocytes. By R. Lemberg, W. H. Lockwood and J. W. Legge . 363

37. Pyruvic dehydrogenase of *Bacterium coli*. By J. L. Still. (With three figures) 380

[P.T.O.]

PAGE

38. Glyoxalase of *Bacterium coli*. By J. L. STILL 390

39. Adenylic deaminase activity in B-avitaminosis and in starvation. By G. D. LU and D. M. NEEDHAM. (With eleven figures) 392

40. The cystine and methionine in certain species of grassland herbage. By A. M. SMITH and T. WANG 404

41. The proteins of *Hevea brasiliensis*. 2. Analysis of a product isolated from crepe rubber. By G. R. TRISTRAM 413

42. The separation of amino-acids by means of their copper salts. 3. The hydrolysis of gliadin. An investigation of the dicarboxylate fraction, including the isolation of *l*-glutamic acid as an hydrolysis product. By B. W. TOWN 417

43. An analysis of Bence-Jones protein. By J. DEVINE . . . 433

INDEX OF AUTHORS

PAGE

Allcroft, W. M. 254
Aschaffenburg, R. 272

Bell, F. O. 294

Crook, E. M. 226
Cunningham, C. N. 219

Devine, J. 433

Folley, S. J. 254

Grant, M. W. 272

Ireland, J. T. 283

Legge, J. W. . . 328, 339, 353, 363
Lemberg, R. . . 328, 339, 353, 363
Lockwood, W. H. . . 328, 339, 363
Lowndes, J. 315
Lu, G. D. 392

Macara, T. J. R. 315

PAGE

Martin, A. J. P. . . . 294

Needham, D. M. 392

Peters, B. A. 219
Plimmer, R. H. A. . . . 315

Rimington, C. . . . 321

Scott Blair, G. W. . . 267
Smith, A. M. 404
Stewart, C. P. 245
Still, J. L. 380, 390
Synge, R. L. M. . . . 294

Thompson, J. C. 245
Town, B. W. 417
Tristram, G. R. 413
Turfitt, G. E. . . . 237

Waller, H. 272
Wang, T. 404

CONTENTS OF VOLUME 35, No. 4

(*All rights reserved*)

PAGE

44. Diffusing factors. 7. Concentration of the mucinase from testicular extracts and from *Crotalus atrox* venom. By J. MADINAVEITIA. (With three figures) 447

45. Studies on diffusing factors. 8. The reduction of the viscosity of vitreous humour preparations by ascorbic acid and some diazo compounds. By J. MADINAVEITIA and T. H. H. QUIBELL. (With two figures) 453

46. Diffusing factors. 9. The effect of salts on the action of testicular extracts on the viscosity of vitreous humour preparations. By J. MADINAVEITIA and T. H. H. QUIBELL. (With one figure) 456

47. Esters of phosphoric acid. 4. Phosphoryl hydroxyamino-acids. By R. H. A. PLIMMER 461

48. On the pH at the surface of ovalbumin molecules, and the protein error with indicators. By J. F. DANIELLI. (With four figures) 470

49. The metabolism of rat liver during carcinogenesis by butter yellow. By J. W. ORR and L. H. STICKLAND. (With one figure) 479

50. The effect of thiamine (vitamin B_1) on fermentation of yeast. By H. LASER. (With six figures) 488

51. The formation of succinic acid in yeast. By A. KLEINZELLER 495

52. Nicotinamide, cozymase and tissue metabolism. By P. J. G. MANN and J. H. QUASTEL. (With two figures) 502

53 The site of action of narcotics in respiratory processes. By H. MICHAELIS and J. H. QUASTEL 518

54. Protein catabolism and protein sparing under adrenaline. By C. REID . 534

55. A simplified estimation of lactate in muscle. By R. E. JOHNSON and A. N. BARGER 538

56. The micro-determination of potassium. By C. R. HARINGTON. (With one figure) 545

57. Estimation of vitamin A and carotene in human blood. By S. YUDKIN. (With one figure) 551

58. Studies in detoxication. 10. The conjugation and oxidation of p-hydroxybenzenesulphonamide in the rabbit. The characterization of p-sulphonamidophenylglucuronide. By H. G. SAMMONS, J. SHELSWELL and R. T. WILLIAMS 557

59. The hydrolytic degradation of the antigenic complex of *Bact. typhosum* Ty 2. By G. G. FREEMAN and T. H. ANDERSON 564

60. The 3:5-dinitrobenzoyl derivatives of the amino-acids and their use in separating the isomers of leucine and valine. By B. W. TOWN 578

61. On the method of formation of pyruvic acid by barley. By W. O. JAMES, G. M. JAMES and A. H. BUNTING 588

[P.T.O.]

CONTENTS

INDEX OF AUTHORS

PAGE

Anderson, T. H. 564

Barger, A. N. 538
Bunting, A. H. 588

Danielli, J. F. 470

Freeman, G. G. 564

Harington, C. R. 545

James, G. M. 588
James, W. O. 588
Johnson, R. E. 538

Kleinzeller, A. 495

Laser, H. 488

Madinaveitia, J. 447, 453, 456

PAGE

Mann, P. J. G. 502
Michaelis, M. 518

Orr, J. W. 479

Plimmer, R. H. A. 461

Quastel, J. H. 502, 518
Quibell, T. H. H. 453, 456

Reid, C. 534

Sammons, H. G. 557
Shelswell, J. 557
Stickland, L. H. 479

Town, B. W. 578

Williams, R. T. 557

Yudkin, S. 551

CONTENTS OF VOLUME 35, Nos. 5 AND 6, 1941

(All rights reserved)

PAGE

62. The enzymic decarboxylation of oxaloacetate. By L. O. KRAMPITZ and C. H. WERKMAN. (With one figure) 595

63. Proliferation-promoting intercellular hormones. 2. Evidence for their production by living cells as a response to injury. By J. R. LOOFBOUROW, SISTER C. M. DWYER and SISTER A. G. CRONIN. (With four figures) 603

64. Enzymic decomposition of amino-sugars. By C. LUTWAK-MANN 610

65. The methylation of wool with methyl sulphate and methyl halides. By S. BLACKBURN, E. G. H. CARTER and H. PHILLIPS. (With two figures) 627

66. The isolation of amino-acids from rubber latex. By G. S. WHITBY and H. GREENBERG 640

67. A colorimetric method for the determination of citrulline. By A. G. GORNALL and A. HUNTER 650

68. Hemicellulose preparation and chlorination cellulose. By I. A. PREECE . 659

69. Histidine in urine. 2. By E. RACKER. (With four figures) 667

70. A method for the micro-estimation of iron in biological materials. By R. H. THORP. (With two figures) 672

71. Biological synthesis of oxaloacetic acid from pyruvic acid and carbon dioxide. 2. The mechanism of carbon dioxide fixation in propionic acid bacteria. By H. A. KREBS and L. V. EGGLESTON 676

72. Cytochrome system in *Bacterium coli commune*. By D. KEILIN and C. H. HARPLEY 688

73. Vitamin A studies with rats and pigs. By R. BRAUDE, A. S. FOOT, K. M. HENRY, S. K. KON, S. Y. THOMPSON and T. H. MEAD. (With one figure) . . 693

74. Metabolism of steroids. 2. The isolation of cholestane-3:5:6-triol and other substances from ox liver extracts. By G. A. D. HASLEWOOD 708

75. The sterol requirements of the larva of the beetle, *Dermestes vulpinus* Fabr. By G. FRAENKEL, J. A. REID and M. BLEWETT 712

76. The use of micro-organisms in sugar analysis. 3. The effects of various salts on the activity of *Proteus vulgaris* in removing glucose and some possible sources of error in its use as a reagent for the estimation of glucose. By T. F. NICHOLSON and R. McG. ARCHIBALD 721

77. Correlation between sex hormones, thyroid hormones and desoxycorticosterone as judged by their effects on the weights of organs of gonadectomized rats. By V. KORENCHEVSKY and K. HALL 726

78. Serum gonadotrophin. 1. Preparation of a stable concentrate from pregnant mare's serum. By C. RIMINGTON and I. W. ROWLANDS. (With four figures) . . 736

[P.T.O.]

CONTENTS

INDEX OF AUTHORS

	PAGE
Archibald, R. McG.	721
Blackburn, S.	627
Blewett, M.	712
Braude, R.	693
Carter, E. G. H.	627
Cronin, A. G.	603
Dwyer, C. M.	603
Eggleston, L. V.	676
Foot, A. S.	693
Fraenkel, G.	712
Gornall, A. G.	650
Greenberg, H.	640
Hall, K.	726
Harpley, C. H.	688
Haslewood, G. A. D.	708
Henry, K. M.	693
Hunter, A.	650
Keilin, D.	688
Kon, S. K.	693
Korenchevsky, V.	726
Krampitz, L. O.	595
Krebs, H. A.	676
Loofbourow, J. R.	603
Lutwak-Mann, C.	610
Mead, T. H.	693
Nicholson, T. F.	721
Phillips, H.	627
Preece, I. A.	659
Racker, E.	667
Reid, J. A.	712
Rimington, C.	736
Rowlands, I. W.	736
Thompson, S. Y.	693
Thorp, R. H.	672
Werkman, C. H.	595
Whitby, G. S.	640

ERRATA

Vol. 34, Nos. 8 & 9, p. 1221, Fig. 3

Note that figures for pectin concentrates on the diagram should be in reverse order as in legend

Vol. 34, Nos. 10 & 11

p. 1419, line 1 of title	*for*	$C_{20}H_{20}O_5Cl$
	read	$C_{20}H_{21}O_5Cl$
p. 1419, line 10 of text	*for*	$C_{20}H_{20}O_5Cl$
	read	$C_{20}H_{21}O_5Cl$
p. 1420, line 39 of text	*for*	$C_{20}H_{20}O_5Cl$
	read	$C_{20}H_{21}O_5Cl$

I. CHANGES IN PHOSPHATE DISTRIBUTION DURING ANAEROBIC GLYCOLYSIS IN BRAIN SLICES

By MARJORIE GIFFEN MACFARLANE and HANS WEIL-MALHERBE
From the Biochemical Department, Lister Institute, London

(*Received 9 November 1940*)

It has been shown that the changes in phosphate distribution during fermentation in the living yeast cell are so consistent with the theory, developed from experiments with cell-free extracts, that anaerobic glycolysis takes place by intermediate phosphorylation that there can be little doubt that the processes are analogous [Macfarlane, 1939]. It was pointed out at the same time that in cells the normal glycolysis may be maintained by a cycle of P transference which involves such small amounts of P that the isolation or detection of phosphorylated intermediate products may be extremely difficult. There has been considerable argument over the existence in certain tissues, e.g. brain, tumour, retina, embryo etc., of a non-phosphorylating type of glycolysis, though the preparation of cell-free phosphorylating extracts from these tissues [Geiger, 1940; Boyland & Boyland, 1935; Kerly & Bourne, 1940; Meyerhof & Perdigon, 1939] supports the view that an analogous phosphorylation takes place in the cells. The fact that glucose can act as a phosphate acceptor in dialysed brain dispersions [Ochoa, 1940] is even more convincing evidence of the correctness of this theory. We have examined the changes in P distribution which take place during anaerobic glycolysis and autolysis in slices of rabbit brain and find that they are consistent with the view that the glycolysis is dependent on phosphorylation.

Methods. The rabbits were killed by a blow on the neck and the brain removed immediately into Ringer solution. Slices were cut in the manner usual for manometric experiments and kept aerobically in Ringer solution at room temperature until sufficient material was obtained. Roughly equal amounts of tissue were drained and placed in tared flasks containing the medium (0·5 g. to 5 ml. medium) and the wet weight of tissue obtained by reweighing. The flasks were immersed in a thermostat at 37° and shaken mechanically. A stream of N_2, containing 5 % CO_2, was passed through for 3 min.; the outlet was then closed and the flasks were left connected to the gas cylinder. Enzyme action was stopped by the addition of trichloroacetic acid in final concentration 3-5 %. Approximately 20-25 min. elapsed between the death of the animal and the start of anaerobic incubation, at which point the zero value was taken. It was anticipated that this method of weighing, though it causes the minimum injury to the slices, would be subject to considerable error owing to occlusion of liquid in bunches of slices; it was found, however, that though there were occasional anomalous values the results of manifold experiments were on the whole in good agreement. The average dry weight was 12·5 % of the wet weight.

The medium used was a phosphate-free modification of Krebs & Henseleit [1932] Ringer solution, saturated with the N_2/CO_2 mixture, the K_2HPO_4 being replaced by an amount of KCl sufficient to give the same ionic concentration of K; for glycolysis it contained in addition 0·2 % glucose. An experiment was performed in which the medium used was horse serum which had been dialysed against P-free and bicarbonate-free Ringer solution. Before use the serum was inactivated by heating for 2 hr. at 56°; bicarbonate was then added in the required amount and the serum saturated with the gas mixture. Contrary to expectation the serum did not decrease the autolysis of the tissue, as manifested by the liberation of phosphate, and its use therefore offered no advantage over Ringer solution. Estimations were made on aliquots of the trichloroacetic acid filtrate as follows: total P after ashing, and inorganic plus creatine P by direct estimation, by Briggs's method; pyrophosphate by calculation from the hydrolysis curve in N HCl at 100°, pyro-P $=\Delta$ (7 min.–0 min.)—Δ (30 min.–7 min.); hexosediphosphate-P by the amount of alkali-labile P formed by incubation with zymohexase (dialysed muscle extract) in presence of 0·1 N KCN at pH 6·8 and 37° for 15 min.; lactic acid by oxidation with ceric sulphate [Gordon & Quastel, 1939] and iodimetric titration of the acetaldehyde-bisulphite compound. Values are expressed in mg./g. dry weight.

Changes in inorganic and pyrophosphate-P during anaerobic glycolysis. Table 1 shows that the inorganic P content of slices incubated anaerobically with glucose for 30 min. was much less than that of slices incubated without glucose, both absolutely and also relatively to the total acid-soluble P, which increased noticeably during autolysis. There was, however, no absolute decrease in inorganic P compared with the initial value and therefore no direct proof of esterification. The observed values were nevertheless quite consistent with the idea that the whole of the P necessary for the glycolytic cycle may be derived from adenylpyrophosphate, and that in the absence of glucose the labile P is broken down with liberation of inorganic phosphate; the fact that after short anaerobiosis brain slices lose their capacity to break down glucose [Dickens & Greville, 1933] affords some support for this view. It was thought possible that re-esterification of inorganic P might be demonstrated by allowing a partial breakdown of the combined P to take place before the addition of glucose. It was found, however, that in the absence of glucose the breakdown of the pyrophosphate-P initially present was so rapid, even at 20°, that it was complete in 3 min. (Table 2), little more than the time considered necessary to secure anaerobic conditions, and that after this time the slices had also lost the ability to form lactic acid (Table 3). In the presence of glucose (Table 2) the initial level of

Table 1. *Anaerobic incubation of brain slices at 37°*

The values obtained for the three experimental conditions, A, B, C, show the extent of variation in aliquots of the same brain or in similar aliquots from different brains. The horizontal line of figures is not necessarily from the same animal in A, B and C.

A Initial				B 30 min. glycolysis				C 30 min. autolysis			
	Inorganic P				Inorganic P				Inorganic P		
Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.	Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.	Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.
2·80	1·52	**54**	3·52	3·12	1·56	**50**	37·2	3·36	2·24	**67**	2·6
2·96	1·60	**54**	3·76	2·96	1·54	**52**	32·5	2·80	1·84	**66**	2·9
2·72	1·60	**59**	—	2·56	1·48	**58**	33·1	3·20	2·24	**70**	—
2·72	1·52	**56**	3·20	2·56	1·36	**53**	37·2	2·96	2·16	**73**	2·7
2·32	1·28	**55**	—	2·88	1·68	**58**	38·8	3·36	2·16	**74**	—
Average:											
2·7	1·5	**55·5**	3·5	2·8	1·5	**54**	36	3·1	2·1	**68**	2·7

Table 2. *Changes in P fractions during incubation at 37°*

Incubation time min.	Glucolysis					Autolysis		
	Total P	Inorganic P	Pyro-P	Lactic acid	$Q_L^{N_2}$	Total P	Inorganic P	Pyro-P
0	2·12	0·98	0·16	3·4	—	2·83	1·39	0·14
3	2·74	1·44	0·17	13·7	51	3·10	1·79	0
10	2·77	1·58	0·06	16·1	19	3·13	1·83	0·01
30	2·88	1·69	0·07	37·2	17	3·34	2·18	0
90	2·74	1·81	0·06	80·8	13	3·38	2·46	0·01

Table 3. *Addition of glucose after preliminary anaerobic incubation for varying times*

		37°				20°			
			Inorganic P				Inorganic P		
	Total time min.	Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.	Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.
No glucose	0	2·69	1·54	**57**	3·2	2·56	1·38	**54**	2·2
,,	3	2·94	1·87	**64**	3·2	2·77	1·76	**63**	2·6
,,	15	2·85	1·91	**67**	3·2	2·58	1·76	**68**	2·2
,,	30	2·99	2·18	**73**	2·4	2·74	1·90	**69**	2·8
Glucose added:									
Initially	30	2·59	1·48	**57**	32·8	2·61	1·41	**54**	16·9
After 3 min.	30	2·83	1·95	**69**	4·8	3·14	1·87	**60**	5·2
After 15 min.	30	3·02	2·10	**70**	3·0	2·88	2·13	**74**	2·6

pyrophosphate-P was maintained for a few minutes and then decreased to a fairly constant level approximately one-third of the original value. There was a concomitant decrease in the rate of lactic acid production, the $Q_L^{N_2}$ falling from a value of 50 over the first few minutes to about 15 over the next hour. The latter is that commonly reported for brain slices, but the equilibration period of 10–20 min. necessary in the manometric technique precludes the observation of a rapid initial glycolysis. The abnormally low initial value of total and inorganic phosphate in this experiment is probably due to the fact that the brain slices were immersed immediately after cutting in glucose-Ringer solution, instead of glucose-free Ringer; it was found on several occasions that this procedure prevented the liberation of some acid-insoluble compound during the aerobic storage of the slices. On the presumption that the pyrophosphate-P is derived from adenylpyrophosphate, the amount of pyro-P available for the formation of hexosephosphoric esters, etc. is of the order of 0·1–0·2 mg. P per g. dry tissue, roughly only 5 % of the total acid-soluble P.

Estimation of hexosediphosphate. From previous experiments with yeast it seemed probable that the ester present in largest amount would be hexosediphosphate and attempts were made to identify this ester using larger quantities of tissue. The experiments were made with brain slices from three or more rabbits (10–20 g. tissue), the trichloroacetic acid filtrates from the individual experiments being pooled for fractionation. The filtrate was adjusted to pH 8 and treated with barium acetate and then with alcohol to 10 % final concentration. The crude Ba precipitate was dissolved in HCl and the Ba removed as sulphate. In most of the experiments attempts were made to remove the inorganic P by addition of $MgCl_2$ and NH_3, but a considerable amount of organic P was carried down with the precipitate; in the third glycolysis experiment (cf. Table 4) the estimation of hexosediphosphate was made without further purification. The accuracy of the method used—enzymic formation of alkali-labile P—was in this case seriously limited by the tenfold excess of inorganic P over organic P; the method is, however, the most specific available and the positive results quoted are outside the experimental error. There was only a poor recovery (30–70 %) of the organic P calculated to be present in the crude Ba precipitates, probably owing to adsorption on the $BaSO_4$ precipitate. Table 4 shows that in two out of the three glycolysis experiments hexosediphosphate was detected; the amounts present, though absolutely very small, were of the order expected from the amount of pyrophosphate available. No hexosediphosphate was found in the autolysed slices and only a trace in the unincubated slices.

Table 4. *Fractionation of acid-soluble P compounds in brain slices*

mg. P/g.

		Autolysis	Glycolysis		
	Initial	20 min.	20 min.	20 min.	15 min.
Total P	2·90	3·33	3·25	3·28	3·28
Inorganic P	1·56	2·16	1·66	1·75	1·84
Pyro-P	0·24	—	—	0·20	0·11
Hexosediphosphate-P	0·01	0	0·03	0·09	0
P in soluble Ba salts	0·80	0·93	0·89	1·02	0·88

The fractions of acid-soluble organic P hydrolysed between the 7th and 180th min., and that not hydrolysed in 180 min., remained almost the same during anaerobic incubation with or without glucose. The amount of P present in compounds with soluble Ba salts was also of the same order in all experiments. It therefore seems unlikely that these compounds are precursors of lactic acid but their nature was not investigated. According to Tschalisow [1932] and Palladin [1934] hexosemonophosphate is present in brain tissue, but the identification of this "lactacidogen" was very unspecific. Booth [1935] has isolated from both sheep and horse brain a compound, identified as a choline ester of sphingosinephosphoric acid, which accounted for at least 50 % of the soluble organic P present in the tissue. The soluble fraction probably includes the nucleotide-P of the coenzymes.

DISCUSSION

It was shown by Engel & Gerard [1936], working with lobster and crab nerve, that during anaerobiosis there is an extensive hydrolysis of both phosphoarginine and adenylpyrophosphate with a corresponding rise in inorganic phosphate; both these compounds were resynthesized by subsequent exposure to O_2. A rapid breakdown of phosphocreatine takes place in mammalian brain [Kerr, 1935]. We have shown in the present experiments that on anaerobic incubation of rabbit brain slices there is a very rapid breakdown of pyrophosphate-P, followed by a further steady liberation of inorganic P from a source previously insoluble; in the presence of glucose there is only a partial breakdown of pyrophosphate and a very slight increase in inorganic P. It is clear that the maintenance of the inorganic phosphate at its original level during glycolysis is not in itself proof of a phosphorylating glycolysis, since the continuous liberation of P is presumably only one aspect of a tissue autolysis which would be largely prevented by the utilization of glucose, irrespective of the mechanism of glycolysis. The correlation however between the rapid loss of glycolytic capacity, observed first by Dickens & Greville [1933], and the rapid breakdown of pyrophosphate, and also between the aerobic restoration of this capacity [Quastel & Wheatley, 1937] and the aerobic resynthesis of adenylpyrophosphate in similar tissue [Engel & Gerard, 1936], affords considerable support for the view that the anaerobic glycolysis in brain tissue takes place through the intermediate formation of phosphoric esters by transference of P from adenylpyrophosphate. This view is strengthened by the detection of hexosediphosphate in the glycolysing tissue. The amount of P involved in the glycolytic cycle in brain tissue with a $Q_L^{N_2}$ about 15 is apparently of the order 0·1–0·2 mg. P per g. dry tissue; in living yeast the corresponding figures are of the order 1–2 mg. P per g. and $Q_{CO_2}^{N_2}$ 100 to 200. The glycolytic rate may therefore afford a rough guide to the amount of P engaged in the cycle.

It appears from the high rate of glycolysis in the first few minutes that the values of $Q_L^{N_2}$ generally obtained in manometric experiments are considerably lower than the maximum glycolytic capacity of the tissue. This is consistent with the occasional observation of an aerobic glycolysis which exceeds the normal anaerobic glycolysis, as in certain experiments with NH_4^+ or maleic acid [Weil-Malherbe, 1938] and with guanidines [Dickens, 1939]. This fact bears on the significance of the Pasteur-Meyerhof quotient, since it is obviously difficult to correlate the aerobic and anaerobic activities of a tissue if the enzyme system is so greatly affected by anaerobiosis.

Geiger [1940] has shown that during glycolysis in brain extracts inorganic phosphate is esterified with formation of hexosephosphoric esters, while adenylpyrophosphate, cozymase and magnesium are essential coenzymes for glycolysis. His main objection to the view that hexosephosphoric esters are intermediaries in the glycolysis is the fact that the rate of lactic acid formation from these esters by brain extract is much lower than that from glucose, and is not accelerated by arsenate. The imbalance between the rates of formation and breakdown of hexosephosphoric esters in cell-free extracts clearly does not exclude phosphorylation as the main path of glycolysis: it is a phenomenon which is characteristic of alcoholic fermentation by yeast juice and one to which we owe the isolation of hexosediphosphate by Harden & Young. It has been shown that the rapid fermentation of phosphopyruvic acid, and therefore of its precursor hexosediphosphate, is dependent on the presence both of glucose (or some other acceptor) and inorganic phosphate [Meyerhof & Kiessling, 1935]. The accelerating action of arsenate is apparently an even more complex process for which a number of explanations have been offered [Needham & Pillai, 1937; Meyerhof *et al.* 1937; Negelein & Brömel, 1939]; it seems that it may be influenced in certain cases by factors not yet understood, for Pillai [1938] observed that, contrary to the usual experience, lactic acid formation from glycogen and hexosediphosphate by his fresh muscle extracts was not accelerated by arsenate, though the rate of dismutation of triosephosphate and pyruvate was increased.

SUMMARY

During anaerobic glycolysis by rabbit brain slices the inorganic phosphate is maintained at its initial level while the content of pyrophosphate-P falls to about one-third of the original. Hexosediphosphate was present in small amounts in the glycolysing tissue. Anaerobic incubation without glucose led to a very rapid disappearance of pyrophosphate-P, with a simultaneous loss of glycolytic capacity. These results are considered to support the view that glycolysis is

maintained by a cycle of P transference involving approximately 0·1–0·2 mg. P per g. dry brain tissue.

One of us (H. W.-M.) wishes to acknowledge the hospitality of the Lister Institute during August and September.

REFERENCES

Booth (1935). *Biochem. J.* **29**, 2071.
Boyland & Boyland (1935). *Biochem. J.* **29**, 1097.
Dickens (1939). *Biochem. J.* **33**, 2017.
Dickens & Greville (1933). *Biochem. J.* **27**, 1138.
Engel & Gerard (1936). *J. biol. Chem.* **112**, 379.
Geiger (1940). *Biochem. J.* **34**, 465.
Gordon & Quastel (1939). *Biochem. J.* **33**, 1332.
Kelly & Bourne (1940). *Biochem J.* **34**, 563.
Kerr (1935). *J. biol. Chem.* **110**, 625.
Krebs & Henseleit (1932). *Hoppe Seyl. Z.* **210**, 33.
Macfarlane (1939). *Biochem. J.* **33**, 565.
Meyerhof & Kiessling (1935). *Biochem. Z.* **281**, 249.
Meyerhof, Kiessling & Schulz (1937). *Biochem. Z.* **292**, 25.
Meyerhof & Perdigon (1939). *C.R. Soc. Biol., Paris*, **132**, 186.
Needham & Pillai (1937). *Biochem. J.* **31**, 1837.
Negelein & Bromel (1939). *Biochem. Z.* **301**, 135.
Ochoa (1940). *Nature, Lond.*, **146**, 267.
Palladin (1934). *Bull. Soc. Chim. biol., Paris*, **16**, 1193.
Pillai (1938). *Biochem. J.* **32**, 1087.
Quastel & Wheatley (1937). *J. biol. Chem.* **119**, 80 *P*.
Tschalisow (1932). *Z. ges. Neurol. Psychiat.* **142**, 85.
Weil-Malherbe (1938) *Biochem. J.* **32**, 2257.

2. METABOLISM OF NORMAL AND TUMOUR TISSUE

19. THE METABOLISM OF INTESTINAL MUCOUS MEMBRANE

By FRANK DICKENS and HANS WEIL-MALHERBE

From the Cancer Research Laboratory, North of England Council of the British Empire Cancer Campaign, Royal Victoria Infirmary, Newcastle-upon-Tyne

(*Received 30 December 1940*)

Studies of the metabolism of intestinal mucous membrane are not numerous and seem to have been restricted to the mucosa of the large intestine [Warburg *et al.* 1924; Rosenthal & Lasnitzki, 1928; Bumm *et al.* 1934]. Dickens & Šimer [1930, 2] found the R.Q. of mucosa from the ileum to be low (0·85), but this result was not considered surprising in view of the low glycolytic activity reported by Warburg *et al.* [1924]. Weil-Malherbe [1938], however, observed with mucosa from rat jejunum a metabolism fundamentally different from that previously recorded for intestinal mucosa. All these various results are summarized in Table 1 from which it is seen that there is an extraordinarily great discrepancy between results obtained by different authors. Thus, while according to Warburg *et al.* mucous membrane belongs to the group of tissues with very low glycolysis, such as liver and kidney, Rosenthal & Lasnitzki as well as Bumm *et al.* found a high anaerobic glycolysis in their experiments with mucosa from the large intestine. Both groups of workers reported however a low aerobic glycolysis of this tissue, though occasionally high values were observed. Weil-Malherbe, on the other hand, found with mucosa from the jejunum very high values of aerobic glycolysis and a practically complete absence of the Pasteur effect. These observations seemed to us important enough to be followed up by a more detailed investigation in connexion with our previous studies on the significance of high aerobic glycolysis

Table 1. *Metabolism of intestinal mucous membrane: published data*

Average figures have been calculated, where possible, from the authors' results.

Part of intestine	Species	Medium*	No. of exp.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	R.Q.	Author
Not stated	Rat	BR	1	12·4	1·6	3·5		Warburg *et al.* [1924]
Large intestine	Rat	BR	9	14·6	4·2	15·1	–	Rosenthal & Lasnitzk [1928]
,,	,,	Serum	2	9·9	1·1	10·3	—	,,
,,	Rabbit	BR	11	−11·1	2·7	14·2	-	,,
,,	,,	Serum	10	−11·2	1·4	12·6	--	,,
Ileum	Rat	PO_4-R	2	− 9·4		–	0·85	Dickens & Šimer [193
Large intestine	Guinea-pig	B & PO_4-R 95% O_2 (10 or 15% O_2)	18 (18)	14·6 (13·2)	4·2 (9·6)	15·7	—	Bumm *et al.* [1934]
Jejunum	Rat	BR	4	−14·6	18·3	20·4		Weil-Malherbe [1938]

* BR = bicarbonate-Ringer solution + glucose.
PO_4-R phosphate-Ringer solution + glucose.
Serum - inactivated serum + glucose.

combined with low R.Q. in tumours [Dickens & Šimer, 1930, 2; 1931, 2]. Our results confirm and extend the earlier observations of Weil-Malherbe on the mucosa of jejunum. They show that this tissue differs in some respects from the mucosa of the large intestine. As in tumour, high aerobic and anaerobic glycolyses are combined with a low R.Q. Moreover, the Pasteur effect is completely absent, even if Ringer solution is replaced by serum.

EXPERIMENTAL

Preparation of tissue. The intestine was first rinsed out with a fine jet of water to remove the greater part of the contents. A length of about 1 in. was then cut open and spread on a glass plate with the mucous membrane facing upwards. After careful blotting with filter paper to remove adhering moisture and solid particles the mucosa was scraped off with the aid of a microscope slide. Another slide served to pin down the muscularis. With practice the mucosa can be stripped off entirely and without laceration. After weighing on a torsion balance the mucous membrane was transferred to the manometer vessel, and another piece was dried immediately for determination of the dry weight/wet weight ratio.

Methods. Measurements of respiration and of aerobic glycolysis at 37·5° were usually carried out simultaneously by Warburg's 2-vessel method in bicarbonate-Ringer [Krebs & Henseleit, 1932] or in inactivated sheep serum. A third manometer, filled with $N_2 + 5\,\%\ CO_2$, served for the measurement of anaerobic glycolysis. If not stated otherwise the medium contained 0·2 % glucose. Retention in serum was determined by the method of Dickens [1939]. Aerobic glycolysis in the 2-vessel method was calculated on the basis of an R.Q. of unity. Measurements of R.Q. were carried out in phosphate-Ringer by the first method of Dickens & Šimer [1930, 1] and in bicarbonate-Ringer or serum by the second method of Dickens & Šimer [1931, 1]. Lactic acid was estimated by the method of Gordon & Quastel [1939].

Infection. Bacterial contamination, noticeable by the development of a faecal smell and a steadily rising metabolism, was encountered only in a few experiments with tissue from the lower part of the ileum or from the large intestine, and even then only in the second hr. of the experiment: such experiments were of course discarded.

Thickness of the tissue. The data collected in Table 2 show that the thickness (moist wt./area) of even the whole intestinal wall of the rat is well below the limiting thickness for complete O_2-saturation, which, for a tissue with $Q_{O_2} = 15$, is 0·47 mm. in pure O_2 and 0·21 mm. in 20 % O_2 [Warburg, 1923]. An adequate diffusion of O_2 was therefore assured in all experiments. The thickness of the mucosa is greatest at the upper end of the intestine and diminishes steadily towards the rectum.

Disintegration of the tissue. A considerable loss of weight is observed after pieces of intestinal mucosa have been shaken in Ringer solution at 37·5° for an hour. This disintegration varies greatly from one experiment to another, even with tissue from the same animal and the same part of the intestine, probably owing to the degree of success in stripping off an unbroken piece of mucosa in its entirety. The loss of wt. is usually higher in the small volume than in the large one, but there is no regularity. Our first experiments, which were carried out before this fact was sufficiently appreciated, were calculated on the final dry wt. as is the routine in tissue metabolism work. The results showed a considerable variation and the figures obtained were sometimes unusually high. Results of

Table 2. *Thickness of the intestinal wall and its components (rat)*

		Thickness in mm.		
		Whole wall	Mucosa	Muscularis
Jejunum		0·095	0·062	0·032
		0·105	0·069	0·024
		0·093	0·061	0·024
	Mean	0·098	0·064	0·027
Ileum		0·086	0·048	0·026
		0·071	0·044	0·023
	Mean	0·078	0·046	0·025
Colon		0·115	0·046	0·059
		0·112	0·023	0·067
	Mean	0·114	0·035	0·063

Table 3. *Loss of weight after 1 hr. shaking at 37·5°*

				% Recovery $\left(\frac{\text{Final dry wt.}}{\text{Initial dry wt.}} \times 100\right)$		
				In $O_2 + 5\%$ CO_2		In $N_2 + 5\%$ CO_2
Species	Tissue	Medium*	Initial dry wt. / Initial wet wt.	$v_F = 2$ ml.	$v_F = 8$ ml.	$v_F = 3$ ml.
Rat	Duodenum, mucosa	BR	0·199	59·0	49·1	64·8
		BR	0·197	48·5	67·5	78·0
Rat	Jejunum, mucosa	BR	0·211	61·6	52·1	61·3
		BR	0·239	68·5	76·0	59·3
		BR	0·226			65·6
		BR	0·198	29·4	44·6	19·6
				25·8	45·6	
				57·0	41·0	
		BR	0·188	49·0	36·0	—
				45·0	51·0	
		BR	0·197	23·0	55·5	61·5
		Serum		100·0	104·5	100·0
		BR	0·195	27·0	70·0	28·0
		Serum		108	60·0(?)	85·0
Rat	Ileum, mucosa	BR	0·193	36·1	38·3	39·4
		BR	0·184	28·4	26·5	31·8
		BR	0·170	27·5	32·4	36·0
		Serum		64·7	56·8	71·6
Rat	Colon, mucosa	BR	0·260	47·5	41·0	48·2
Rat	Jejunum, muscularis	BR	0·227	82·5	68·0	45·8
Rat	Jejunum, whole wall	BR	0·228	91·8	88·5	83·5
Mouse	Jejunum, whole wall	BR	0·234	90·5	89·8	78·0
		Serum		95·4	101	94·8

* BR = Bicarbonate-Ringer solution + glucose.

greater uniformity were obtained when the calculation was based on the initial dry/wet weight ratio. This method was used in most of the experiments to be reported; only a few of the earlier experiments have been retained where the differences within one and the same experiment serve to illustrate a point. Sometimes the results have been calculated by both methods to demonstrate the effect of the loss of weight on the calculation; it will be noticed that the figures based on the final dry weight are up to 5 times higher than those based on the initial dry/wet weight ratio.

In conformity with the decreasing thickness of the mucous membrane the disintegration is greater with material from the ileum and colon than with that from the jejunum.

There is very little loss of weight when the whole intestinal wall is used. If serum is used instead of Ringer solution the disintegration of the mucous membrane is greatly reduced; indeed the mucosa of the jejunum may be recovered almost intact after a 1 hr. experiment.

Results

Examples of the metabolism of the mucous membrane from different parts of the intestine of the same animal are given in Table 4 (cf. also Table 6). The metabolism is most active in the upper and middle parts of the jejunum though respiration was occasionally found highest in the duodenum. Usually respiration is steadier than glycolysis which is very unstable and falls to the same extent aerobically and anaerobically. This fall cannot be due to a mere mechanical breaking up of the tissue as it is also observed in serum where disintegration is

Table 4. *Metabolism of different parts of the intestine in bicarbonate-Ringer*

		Results based on initial dry wet wt.			Results based on final dry wt.		
	Min.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
		Exp. 1. Rat, mucous membrane					
Duodenum	0– 20	8·8	11·1	10·4	21·2	23·0	13·3
	20– 40	- 8·1	7·8	6·9	17·6	16·2	8·8
	40– 60	6·0	4·7	1·9	12·1	9·6	6·2
Jejunum (upper part)	0– 20	15·6	16·5	16·4	23·1	24·0	27·8
	20– 40	14·8	12·3	13·3	22·1	17·9	22·4
	40– 60	11·9	7·0	8·9	16·8	10·1	15·0
Ileum (lower part)	0– 20	5·3	5·5	4·0	17·6	19·4	12·5
	20– 40	- 2·1	2·0	2·2	- 7·3	7·0	6·8
	40– 60	4·6	1·0	1·0	5·5	3·6	3·0
Colon	0– 20	- 3·4	5·2	4·4	6·4	11·0	9·0
	20– 40	- 3·2	3·5	1·9	5·0	3·2	4·0
	40– 60	- 1·4	2·1	0·6	- 2·5	4·3	1·1
		Exp. 2. Rat, mucous membrane					
Duodenum	0– 60	—			- 50·0	24·6	38·5
	60–120		—	—	28·0	7·3	12·6
Jejunum	0– 60				- 22·8	35·1	39·0
	60–120	—			13·8	11·1	18·5
Ileum	0– 60	—		—	13·7	14·3	15·7
	60–120	—		—	10·6	11·0	11·0
Colon	0– 60	—	—	—	- 16·2	7·6	23·4
	60–120	—	—	—	- 7·4	4·7	—
		Exp. 3. Mouse, whole intestinal wall					
Duodenum	0– 60		—	—	- 16·6	20·6	13·0
	60–120		—	—	- 13·3	15·3	9·9
Jejunum	0– 60	—	—	—	28·2	32·1	23·3
	60–120		—	—	- 21·0	23·0	15·9
Ileum	0– 60	—	—	—	- 10·7	4·5	9·3
	60–120	—	—	—	- 10·8	4·4	6·3
Colon	0– 60	—	—	—	- 14·1	3·9	8·1

small. With mucosa from the ileum and colon, respiration, too, is unstable and this may well be ascribed to the greater disintegration of this tissue. If based on the initial dry weight the whole metabolism of these latter tissues is very low.

Respiration and aerobic and anaerobic glycolyses have about equal Q-values, especially if the calculation is based on the initial dry weight. This is at least true for the jejunum, whereas in the cases of the ileum and the colon the results are less consistent. Sometimes the glycolysis was as high aerobically as anaerobically (Table 4, exp. 1 for ileum and colon, exp. 2 for ileum); at other times the anaerobic exceeded the aerobic glycolysis (Table 4, exp. 2 for colon, Table 6 for ileum). In some experiments it was even observed that the glycolysis was higher aerobically than anaerobically, especially in the experiments with the whole intestinal wall of both rat and mouse (Tables 4–6). This is probably an artefact due to a more rapid autolysis under anaerobic conditions [cf. Macfarlane & Weil-Malherbe, 1941].

The high aerobic glycolysis of the isolated mucous membrane is not caused by the severance from its matrix as has been suggested for retina [Warburg *et al.* 1924]. If the whole intestinal wall is examined, the same type of metabolism is found as with the isolated mucosa. In fact the metabolism of the whole intestinal wall almost equals the average of the metabolisms of the isolated mucosa and muscularis (Table 5). In the higher regions of the intestine the metabolism of the whole wall is determined by that of the mucous membrane owing to the higher metabolism and greater weight of the latter. The metabolism of the whole wall of mouse jejunum, especially, greatly resembles that of the isolated mucosa. In the lower regions of the intestine the influence of the muscular coat may be expected to be more marked owing to the decreasing thickness and low metabolism of the mucous membrane.

Table 5. *Metabolism of mucosa, muscularis and whole wall of the upper part of the jejunum of the rat*

All results calculated on initial dry wet weight basis.

	Min.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
Mucosa	0–20	−11·3	14·8	12·6
	20–40	−8·7	8·2	5·6
	40–60	−4·8	3·7	4·0
Muscularis	0–20	−6·3	4·8	6·8
	20–40	−4·5	3·2	4·7
	40–60	−3·9	2·4	4·0
Whole wall	0–20	−7·8	10·6	8·1
	20–40	−5·4	8·7	6·6
	40–60	3·2	8·0	4·9

There is very little loss of weight during an experiment with whole intestinal wall and it therefore does not matter by which method the results are calculated as a comparison of the experiments published in Tables 4 and 6 will show.

Metabolism in serum. According to Negelein [1925] the aerobic glycolysis of normal growing cells such as embryo and the germinal epithelium of testis disappears in serum. This is not the case with intestinal mucosa or whole intestinal wall where aerobic glycolysis persists in serum to at least the same extent as in Ringer solution (Table 6). This result is similar to that obtained previously with the medulla of kidney [Dickens & Weil-Malherbe, 1936, 1] contrary to György *et al.* [1928].

Table 6. *Metabolism of intestine in bicarbonate-Ringer solution and in sheep serum*

All results based on initial dry/wet weight ratio.

		In serum			In Ringer		
	Min.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
Jejunum, mucosa (rat)	0–20	−16·3	25·2	22·9	−10·3	13·3	13·5
	20–40	−15·4	17·7	13·6	−9·9	11·9	9·8
	40–60	−12·9	14·0	10·6	−8·4	9·7	8·4
Ileum, mucosa (rat)	0–20	−10·8	8·5	11·7	−2·1	4·4	7·7
	20–40	−5·0	3·1	4·7	−1·2	2·5	5·8
	40–60	−4·7	1·6	1·4	−1·6	1·5	4·0
Jejunum, whole wall (mouse)	0–20	−15·5	25·0	10·9	−19·0	25·8	10·6
	20–40	16·5	20·8	6·6	−15·8	23·2	8·7
	40–60	−14·6	19·2	6·4	−14·8	18·9	7·5

Table 7. *Chemical control of lactic acid formation*

Jejunal mucosa of the rat.

			µl. lactic acid		
Medium*	Gas phase	Time min.	Manometrically	Chemically	% Chemic./manom.
BR	$N_2=5\%\ CO_2$	120	251	197	78·5
Serum		120	238	206	86·5
Serum		120	460	471	102·5
Serum		150	226	227	100
BR	$O_2-5\%\ CO_2$	150	560	447	80
BR		150	687	484	70·4
BR		150	643	553	86

Table 8. *Effect of substrate on metabolism of mucous membrane of rat jejunum*

		Q_{O_2}					$Q_G^{N_2}$				
Medium*	Min.	No substrate	0·2% glucose	0·2% glycogen	Q_{CO_2} No substrate	Apparent R.Q.	No substrate	0·2% glucose	0·2% glycogen	0·2% fructose	M/100 hexosemonophosphate
					Results based on final dry weight						
BR	0–30	−30·9	—	—	31·0	1·00	8·7	23·6	22·6		
	30–60	−14·6	—	—	11·0	0·75	6·3	17·2	16·8		
	60–90	−12·5	—	—	7·4	0·59	5·0	10·6	11·0		
	90–120	−11·0	—	—	6·8	0·61	5·0	8·2	9·8		—
PO₄-R	0–30	−26·4	−22·2	−21·6	—	—	—	—	—	—	—
	30–60	−23·7	−22·8	−23·7	—	—	—	—	—	—	—
	60–90	−19·7	−19·1	−19·4	—	—	—	—	—		—
	90–120	−20·8	−20·1	−19·5	—	—	—	—		—	—
BR	0–10	—	—	—	—	—	11·4	48·0	26·1	24·0	13·2
	10–30	—	—	—	—		7·0	27·7	15·2	14·7	12·9
	30–60	—	—		—	—	7·6	16·6	12·8	10·4	12·8
	60–90	—	—	—	—	—	8·2	9·8	8·2	9·0	8·8
					Results based on initial dry weight						
BR	0–20	—	—	—	—	—	9·7	9·4	9·8	9·3	9·0
	20–40	—	—	—	—	—	5·4	6·1	6·2	5·7	4·5
	40–60	—	—	—	—	—	3·5	5·6	5·0	4·4	3·7
BR	0–20	—	—	—	—	—	7·3	7·7	11·0	4·7	5·3
	20–40	—	—	—	—	—	4·6	6·7	5·2	3·9	3·4
	40–60	—	—	—	—	—	2·6	4·7	3·2	2·7	2·8

* BR = bicarbonate-Ringer solution.
PO₄-R = phosphate-Ringer solution.

Nature of acid formation. That the acid formation by the intestinal mucosa is really due to glycolysis is shown by the fact that 70–100 % of the acid estimated manometrically is accounted for by chemical estimation of lactic acid (Table 7) and secondly by the disappearance of aerobic and, to some extent, anaerobic acid formation in absence of glucose or another suitable substrate (Table 8). Respiration, on the other hand, is not affected at all by the absence of a substrate.

The effect of addition of substrate on the anaerobic glycolysis seems to be variable. In experiments which were performed in early spring we repeatedly observed a strong effect of glucose and glycogen on the anaerobic acid formation which was increased 3–4 times. When we tried to repeat these experiments in November the only effect was a delay in the fall of glycolysis.

Respiratory quotient. For a strongly glycolysing tissue such as the intestinal mucous membrane one might have expected an R.Q. of about unity. In fact the R.Q. is low and decreases towards the ileal end of the jejunum (Table 9).

Table 9. *Respiratory quotient of mucosa from rat jejunum*

Medium*		Time min.	Q_{O_2}	$Q_G^{O_2}$	R.Q.
		Based on final dry weight			
PO$_4$-R		150	−13·4	—	0·92
PO$_4$-R		150	−16·6	—	0·89
		100	−28·5	—	0·86
		150	−20·8	—	0·84
		Based on initial dry/wet weight ratio			
BR	Duodenal end	150	− 5·7	7·0	0·93
	Middle part	150	5·6	8·6	0·86
	Ileal end	150	−12·5	9·7	0·78
Serum		90	− 5·4	9·1	0·89
		90	− 7·4	10·3	0·83

* BR = bicarbonate-Ringer solution + glucose.
PO$_4$-R phosphate-Ringer solution + glucose.

Effect of acetylcholine. In view of the work of Deutsch & Raper [1936; 1938] on the effect of acetylcholine on the *in vitro* metabolism of the salivary glands of the cat it was of interest to examine whether this substance would cause a similar stimulation of metabolism in intestinal mucous membrane, this being largely a glandular tissue controlled by sympathetic and parasympathetic impulses. Experiments were carried out with acetylcholine (1–5μg./ml.), with eserine (20μg./ml.), with atropine (40μg./ml.) and with acetylcholine + eserine. No effect was observed whether the addition was made before or during the experiment.

Nucleic acid-phosphorus. It has been suggested by Berenblum *et al.* [1939, 1] that the measurements of tissue metabolism should be related to the content of nucleic acid rather than to the dry weight. For the conversion of our figures to the nucleic acid standard we have estimated the nucleic acid-P in intestinal mucosa by the method of these authors. We found the following values:

		Nucleic acid-P, % of dry weight
Jejunum:	Proximal part	0·34
	Distal part	0·40
Ileum:	Proximal part	0·44
	Distal part	0·53

Discussion

It is surprising that the exceptional features of the metabolism of intestinal mucous membrane should have been entirely overlooked by previous workers in the field of tissue metabolism. For some reason their attention was turned almost exclusively on the mucosa of the large intestine, yet the experimental difficulties and hence the variation of the results are much less with jejunal mucous membrane which is thicker, less fragile and free from bacteria. It can easily be stripped off from the smooth surface of the small intestine, whereas the surface of the large intestine is undulated owing to ridges of longitudinal muscle fibres which make it difficult to strip off the mucosa without laceration.

The mucous membrane of the jejunum shows the highest metabolic activity of all parts of the intestinal tract. It belongs to the most actively respiring tissues of the body. Its glycolysis in nitrogen is at least of equal activity. Yet this active glycolysis is not depressed by the respiration; it persists at the same rate in oxygen. This complete absence of the Pasteur effect is the most remarkable and indeed unique property of this tissue. There is no reason to assume that the high aerobic glycolysis which we constantly observed is an artefact which does not exist *in vivo*, since it is due neither to the separation from the intestinal wall, nor to incomplete O_2-saturation, nor to bacterial contamination, nor to a toxic effect of saline, nor to a sudden stimulus caused by liberation of acetylcholine.

Although we have never observed a glycolysis which was smaller aerobically than anaerobically in jejunal mucous membrane, the converse was occasionally seen. This was probably due to a more rapid autolysis under anaerobic conditions. It has been shown for brain tissue that there is a rapid fall of the P_2O_7 fraction anaerobically even in presence of glucose with a corresponding fall of glycolysis [Macfarlane & Weil-Malherbe, 1941].

The metabolism of the isolated mucosa of the ileum and colon is low, especially if allowance is made for the disintegration of the tissue during the experiment. Though it was sometimes observed by ourselves as well as by earlier workers that the aerobic glycolysis reached the level of the anaerobic glycolysis, a definite Pasteur effect was found in other experiments. Some of our experiments with colonic mucous membrane agree in fact very well with the figures of Rosenthal & Lasnitzki [1928]. This variation does not seem to recommend the colonic mucous membrane as a suitable subject for the study of the Pasteur effect. Yet it was used for this purpose by Bumm *et al.* [1934] who claimed to have shown that if the O_2-tension is lowered to 10% the Pasteur effect is inhibited without respiration being affected (cf. Table 1).

As we have previously pointed out [Dickens & Weil-Malherbe, 1936, 1, 2] a high aerobic glycolysis is not specific for either malignant or normal growth. On the other hand a tendency has recently been noticeable [Berenblum *et al.* 1939, 2] to dissociate the high aerobic glycolysis completely from the tumour problem. In experiments to be published one of us has observed examples where there is little change of the metabolism of a normal tissue with low glycolysis during its transformation to a benign tumour or to a precancerous condition, while the development of malignancy is accompanied by a definite increase in the aerobic and anaerobic glycolysis. Thus while aerobic glycolysis is not limited to tumours —and as the present paper shows may even reach the level of the anaerobic glycolysis in a normal 'resting' tissue—it is not correct to assume that the glycolysis of tumour is always merely that of the tissue of origin.

Formerly tumour was considered to be the only tissue where a high glycolysis was accompanied by a low R.Q. We interpreted this [Dickens & Šimer, 1930, 2] as meaning a defective carbohydrate metabolism, and regarded it as a specific property of tumours. Meanwhile, Bywaters [1937] showed that synovial membrane has a similar type of metabolism, but the low activity of this tissue makes comparison difficult. The mucous membrane of the intestine provides another and more satisfactory example, showing clearly that combination of active glycolysis with low R.Q. is not a specific property of tumours. Another interesting similarity is the occurrence of aminoethylalcohol phosphoric ester in both tumour [Outhouse, 1937] and intestine [Colowick & Cori, 1939].

SUMMARY

The metabolism of mucous membrane from the various parts of the intestine has been measured in Ringer solution and in serum. It is found that the jejunal mucous membrane possesses a high respiration, a high glycolysis, aerobically and anaerobically, and a low R.Q.; acetylcholine was without effect. Jejunal mucous membrane is unique among tissues hitherto studied in that the Pasteur effect is absent. These results are not due to toxic action of Ringer solution, as they are also observed in serum; nor are they due to severance of the mucosa from the matrix as the metabolism of the whole intestinal wall is that expected from the summation of its parts.

The metabolism of mucous membrane from ileum and colon is less active and more variable, probably owing to greater fragility. It resembles in general that of the jejunum, but the Pasteur effect was often evident.

These results are discussed in relation to the problem of tumour metabolism.

REFERENCES

Berenblum, Chain & Heatley (1939, 1). *Biochem. J.* **33**, 68.
— — — (1939, 2) *Amer. J. Cancer*, **38**, 367.
Bumm, Appel & Fehrenbach (1934). *Hoppe-Seyl. Z.* **223**, 207
Bywaters (1937). *J. Path. Bact.* **44**, 247.
Colowick & Cori (1939). *Proc. Soc. exp. Biol. N.Y.*, **40**, 586.
Deutsch & Raper (1936) *J. Physiol.* **87**, 275.
— — (1938). *J. Physiol.* **92**, 439.
Dickens (1939). *Biochem. J.* **33**, 230.
— & Šimer (1930, 1). *Biochem. J.* **24**, 905.
— — (1930, 2). *Biochem. J.* **24**, 1301.
— — (1931, 1). *Biochem. J.* **25**, 973.
— — (1931, 2). *Biochem. J.* **25**, 985.
— & Weil-Malherbe (1936, 1). *Biochem. J.* **30**, 659.
— — (1936, 2). *Nature, Lond.*, **138**, 125.
Gordon & Quastel (1939). *Biochem. J.* **33**, 1332.
Gyorgy, Keller & Brehme (1928). *Biochem. Z.* **200**, 356.
Krebs & Henseleit (1932). *Hoppe-Seyl. Z.* **210**, 33.
Macfarlane & Weil-Malherbe (1941). *Biochem. J.* **35**, 1.
Negelein (1925). *Biochem. Z.* **165**, 122.
Outhouse (1937). *Biochem. J.* **31**, 1459.
Rosenthal & Lasnitzki (1928). *Biochem. Z.* **196**, 340.
Warburg (1923). *Biochem. Z.* **142**, 317.
— Posener & Negelein (1924). *Biochem. Z.* **152**, 309.
Weil-Malherbe (1938). *Biochem. J.* **32**, 2257.

3. THE INFLUENCE OF DIETARY FIBRE ON SECRETORY ACTIVITIES OF THE ALIMENTARY TRACT

OBSERVATIONS ON FAECAL PHOSPHATASE EXCRETION AND CALCIUM AND NITROGEN BALANCES OF RATS

By JOHN DUCKWORTH and WILFRED JOHN GODDEN

From the Rowett Institute, Aberdeen

(*Received 14 November 1940*)

Some years ago, during an investigation of the influence of rate of growth on the development of rickets in sheep, parallel studies were undertaken using the rat. Owing to the difficulty of obtaining from rats blood samples sufficiently large for phosphatase determination, and having in mind the observations of Heymann [1930; 1931; 1933] on the faecal excretion of phosphatase in rickets, the concentration of this enzyme in the faeces of the rats was determined. It was observed that wide fluctuations occurred in the elimination of phosphatase when animals were transferred from one diet to another. This was especially noticeable in the case of weanling rats transferred to stock diets. The tentative conclusion was drawn that the augmentations in excretion were caused by increased bulk of ingesta which, it was felt, must increase secretory activity within the alimentary tract. The large increases observed for the weanlings were, no doubt, the result of the sensitivity to stimulus of an immature tract accustomed to a diet mainly of milk. These results were not published.

More recently investigations have been in progress in which domestic animals received larger quantities of fibre in the diet than is customary, and certain questions regarding the influence of bulk of ingesta on activities within the alimentary tracts were raised. The connexion between this problem and earlier investigations led to the undertaking of the present study.

Experimental

Six virgin female albino rats aged 105–140 days and weighing 199–225 g. were housed in pairs in three Hopkins metabolism cages which permit of the separate collection of urine and faeces. The animals were fed daily at 10 a.m. after collection of the urine, faeces and unconsumed food. Food and distilled water were offered in excess of appetite. The composition of the low fibre basal diet is recorded in Table 1.

Table 1. *Low fibre diet*

	g.		g.
Sugar	250	$NaHCO_3$	28
Casein	800	$FeSO_4.(NH_4)_2SO_4.6H_2O$	20
Starch	2200	KH_2PO_4	108
Cottonseed oil	250	NaCl	20
Yeast	160	$MgSO_4.7H_2O$	20
$CaCO_3$	100	Radiostoleum	4 ml.
KCl	40		

The experiment was divided into five periods, the first of 10 days' duration and the remainder of 6 days each. During period 1 the basal diet was offered. During period 2 the basal diet was supplemented with 10 % of acid-washed Ca-free paper pulp. In period 3 20 % and in period 4 30 % of paper pulp was added. Period 5, in which 40 % of paper pulp was added to the basal diet, ended abruptly owing to failure of appetite. The day's ration was made up in a stiff dough to minimize scattering and selective feeding.

There were no intervals for adjustment between the periods, the balances being continuous.

Methods of analysis

Calcium. Samples of urine and faeces were dried and ignited at a dull red heat until all traces of carbon had disappeared. The ash was dissolved in hot dilute HCl and made up to known volume. Aliquots were transferred to conical centrifuge tubes, made just alkaline with dilute NH_4OH and then just acid with dilute CH_3COOH using methyl red as an indicator. The tubes were placed in a boiling water bath and, after a few minutes, an excess of a boiling solution of 4 % $(COONH_4)_2$ was added. The tubes were then held at about 70° for 1 hr. to induce the formation of granular precipitates and allowed to stand at room temperature overnight. The precipitates were centrifuged and washed with 2 % NH_4OH. Supernatant liquors were removed by siphoning to avoid loss of precipitate. The oxalate, dissolved in dilute H_2SO_4, was titrated with permanganate.

Nitrogen. All estimations were performed by the macro-Kjeldahl method.

Phosphatase. The day's faeces were well mixed and a sample taken. The sample was placed in a bottle with 200 ml. distilled water and a little toluene and shaken in an end-over-end shaker for about 4 hr. by which time disintegration was complete. The sample was left at room temperature overnight and then analysed according to Bodansky's [1933] method for serum phosphatase. All results are expressed as Bodansky units.

Results

The Ca balances are recorded in Table 2 and the N balances in Table 3. In Table 4 are given the daily phosphatase excretions in the faeces and the units of phosphatase per g. of fresh faeces. All values given are for the group as a whole.

The animals remained in good health throughout the experiment. During period 1 no diarrhoea occurred but occasionally large, moist boli were passed. Throughout the experiment body weight increased from a mean value of 203 g. at the beginning to a mean value of 229 g. at the end. A certain amount of this increase can be attributed to greater weight of intestinal contents during the periods of high fibre intake.

The inclusion of increasing amounts of fibre caused progressive reductions in the intakes of basal ration. These reductions were moderate, the addition of the maximal quantity of fibre (30 % in period 4) causing a fall of about 20 % in the basal diet intake from the period 1 level.

Urinary Ca values were low, only about 1 % of ingested Ca being excreted by the kidneys. There were slight erratic variations in output, but the range was not wide and the mean excretions for each period were unrelated to changes in fibre intake. The major portion of the Ca was found in the faeces. The balances were positive throughout, the best retention occurring during the period when 10 % of fibre was included in the diet. In periods 3 and 4 the retentions were about the same and, in magnitude, about two-thirds of the period 2 retention.

Table 2. *The effect of adding to the diet increasing amounts of fibre on the daily Ca balances of rats*

Period	Day	Ca intake mg.	Ca output mg.	Ca balance mg.	Ca in urine mg.	Ca in faeces mg.
1	1	1040	1003	+ 37	10·7	992
	5	983	867	+116	6·3	861
	6	1026	1081	− 55	5·4	1076
	7	1004	921	+ 83	7·6	913
	8	1000	922	+ 78	8·4	914
	9	971	982	− 11	6·7	975
	10	913	939	− 26	6·2	933
2	1	960	961	− 1	7·1	954
	2	960	—	—	—	—
	3	1074	953	+121	7·4	946
	4	1002	888	+114	8·2	880
	5	944	888	+ 56	9·3	879
	6	880	835	+ 45	7·1	828
3	1	851	839	+ 12	6·6	832
	2	827	788	+ 39	5·6	782
	3	848	817	+ 31	5·3	812
	4	814	771	+ 43	5·4	766
	5	829	788	+ 41	9·7	778
	6	913	799	+114	7·9	791
4	1	794	853	− 59	6·1	847
	2	795	707	+ 88	5·5	702
	3	815	712	+103	6·9	705
	4	731	697	+ 34	6·4	691
	5	836	782	+ 54	7·9	774
	6	755	723	+ 32	11·4	712
			Averages			
1	—	991	959	+ 32	7·3	952
2	—	972	905	+ 67	7·8	897
3	—	847	800	+ 47	6·8	794
4	—	788	746	+ 42	7·4	738

Urinary N values declined from period to period as the N intake fell. The faecal excretion increased from period 1 to period 2 and again in period 3. The value found for period 4 was somewhat below the period 2 level. The mean retentions were positive in all periods, higher retentions being found in periods 1 and 3 and the lowest in period 4. Negative daily balances preponderated in period 4 but were absent in period 1. Occasional negative daily balances occurred during periods 2 and 3.

During period 1 there were moderate fluctuations in the daily excretion of phosphatase but, although the range was wider than might have been expected, the inclusion of 10 % of fibre in the diet caused a large increase in excretion well beyond the upper limit of period 1 range. In period 3 the excretion of phosphatase was again greater than in period 2 and the values for period 4 were higher than in period 3. The increases with each addition of fibre were progressively less. The mean values showed a more than sevenfold increase between periods 1 and 4. The units of phosphatase excreted per g. of fresh faeces increased in periods 2 and 3 over period 1, but in period 4 the value fell to a point midway between the two extremes. In each of the fibre periods the sudden increase in fibre intake led to maxima in faecal phosphatase excretion, after which the animals tended to adjust themselves to the new conditions.

Table 3. *The effect of adding to the diet increasing amounts of fibre on the daily N balances of rats*

Period	Day	N intake mg.	N output mg.	N balance mg.	N in urine mg.	N in faeces mg.
1	1	3016	2745	+271	2392	353
	5	2852	2544	+308	2260	284
	6	2979	2720	+257	2400	320
	7	2914	2806	+108	2504	302
	8	2901	2660	+241	2350	310
	9	2930	2655	+275	2349	306
	10	2654	2604	+ 50	2328	276
2	1	2743	2436	+307	2092	344
	2	2744	2607	+137	2264	343
	3	3101	3015	+ 86	2665	350
	4	2876	2563	+313	2250	313
	5	2513	2634	−121	2278	356
	6	2312	2575	−263	2272	303
3	1	2458	2223	+235	1738	485
	2	2388	2430	− 42	2090	340
	3	2450	2242	+208	1900	342
	4	2391	2289	+ 62	1990	299
	5	2393	2216	+177	1822	394
	6	2640	2464	+176	2130	334
4	1	2281	2292	− 11	1950	342
	2	2286	2188	+ 98	1839	349
	3	2347	2387	− 40	2062	325
	4	2095	2155	− 60	1870	285
	5	2408	2213	+195	1906	307
	6	2166	2213	− 47	1874	339
			Averages			
1	—	2891	2676	+215	2369	307
2	—	2715	2638	+ 77	2303	335
3	—	2447	2311	+136	1945	366
4	—	2264	2241	+ 23	1917	324

Table 4. *The effect of adding to the diet increasing amounts of fibre on the daily faecal excretion of phosphatase by rats*

Period	Day	Total faecal phosphatase	Phosphatase per g. of faeces	Period	Day	Total faecal phosphatase	Phosphatase per g. of faeces
1	1	182	14·4	3	1	1061	24·2
	5	149	15·0		2	1178	27·4
	6	186	15·4		3	1433	29·9
	7	165	15·6		4	1230	29·0
	8	239	22·0		5	1286	27·8
	9	292	24·6		6	1074	24·8
	10	200	18·5				
Mean		202	17·9	Mean		1210	27·7
2	1	637	28·5	4	1	1598	23·4
	2	1033	33·7		2	1589	22·3
	3	1010	31·5		3	1436	20·8
	4	676	24·4		4	1423	22·0
	5	870	29·0		5	1513	22·2
	6	535	22·9		6	1306	19·3
Mean		794	28·3	Mean		1478	21·7

DISCUSSION

It is felt that the preliminary nature of the observations of the effect of large quantities of crude fibre in the diet on phosphatase excretion in the faeces should be emphasized. At the present time an exact interpretation of the results is not possible because neither the individual contribution of the different secreting organs nor the rates of destruction of the secreted enzyme within the lumen of the intestine during the different periods were assessed. Nevertheless certain conclusions can profitably be drawn.

While it is possible that there was a proportional increase of all secretions, amounting to about 700% in period 4 as compared with period 1, it is more probable that there were greater increases in the secretions of certain organs and smaller, or even no, increases in the case of others. Also, there is no information regarding the possible exhaustion, or semi-exhaustion, of the enzyme within an organ suddenly called upon to increase secretion. In such a case the phosphatase value of the faeces would be, by underestimation, an imperfect guide to measurement of increased secretory activity. Although there is no clear evidence that bulk of ingesta has any marked effect in increasing the speed of propulsion of material through the intestinal tract, if this does occur the faecal phosphatase value would overestimate increased secretory activity owing to reduced opportunity for enzyme destruction.

In the present investigation an objection might be raised to comparing period 1 with period 4 on the ground that this involves the assumption that results found for the fibre-free period represent the normal state of affairs and can therefore be accepted as basic in subsequent discussion. But even if this period be discarded and period 2, with the 10% fibre intake, be accepted as normal, period 4 still exhibits an increase of 100% in faecal phosphatase excretion. If, however, the balance data for Ca and N can be taken as a guide there is no evidence of dysfunction during period 1. Moreover, beyond the occasional appearance of moist boli, defecation was normal.

The main source of the increases in faecal phosphatase excretion is probably the wall of the intestine, since the greater bulk calls for more lubrication during propulsion. In this connexion the distribution of phosphatase within the faecal boli was examined and it was found that the concentration was greater in the pellicle than in the centre. This difference could only have arisen after the boli had been formed and was therefore attributable to the secretory activity of the large intestine. During the periods when 10 and 20% of fibre were added to the diet the quantity of phosphatase per g. of faeces fell within the same range for both periods and the average values were closely similar. In period 4, which represents with an intake of 30% of fibre about the limit that the rat will tolerate, the excretion per g. of faeces fell. This coincides with the concentration of dietary fibre which Westerlund [1938, 2] found increased the faecal Ca output in the rat when fed on a very low Ca diet. The phosphatase data suggest that a 30% level of fibre reduces the efficiency of the intestine in relation to the bulk of ingesta although the total activity is further increased (from the period 2 level). This impairment of efficiency is also revealed in Westerlund's experiment, where Ca intake was very low. As is discussed below this does not occur with diets of normal Ca content.

Several workers have studied the influence of dietary fibre on Ca retention. Bloom [1930] found that the addition of 8% of fibre to a diet of normal Ca content had no effect upon Ca retention of rats, and this was confirmed by Adolph *et al.* [1938] who, however, used a low Ca diet. Ascham [1930–31] considered that bulk may increase faecal Ca losses in dogs but felt that her results might be inconclusive. Conversely, Westerlund [1938, 1, 2] reported that the incorporation of paper pulp or acetone-extracted, electrodialysed straw flour beyond the level of 20% increased the faecal elimination of Ca by rats subsisting on a diet extremely low in Ca and P. It was considered that the increased bulk stimulated secretory activities within the intestine and that an increased rate of transit of the ingesta through the intestine prevented complete reabsorption of the Ca of the secretions, and the conclusion was drawn that Ca requirement was increased. As stated above, there is no good evidence that increased bulk promotes more rapid propulsion of ingesta, and Westerlund states that he had previously failed to establish any relation between Ca absorption and crude fibre consumption in dairy cows. But, as is discussed below, faecal Ca studies and balance data cannot measure total absorptive processes; only net values can be derived. Morgan [1934], studying adult human subjects consuming coarsely ground cellulose added to a standard diet, found that Ca balances tended to become negative during the high fibre period. Sjollema [1923] observed that increased fibre consumption resulted in negative Ca balances in the rabbit where balances previously had been positive. It is generally accepted that the rabbit is an unsatisfactory animal for Ca balance studies owing to the erratic nature of the results yielded. This peculiarity of the rabbit is well shown by the wide variations in blood Ca frequently observed and by the observation of Cowell [1937] that the rabbit excretes considerable quantities of Ca through the colon. This last characteristic is the opposite of that found by Nicolaysen [1934] and Christiansen [1936] for dogs and by McCance & Widdowson [1939] for man.

The Ca balance results of the present study agree with those of Bloom [1930] and of Adolph *et al.* [1938]. But the results can only be adequately interpreted when examined in conjunction with the phosphatase values. Although it was reasonable to expect that increased bulk of ingesta would induce greater secretory activity within the alimentary tract, it was not expected that such increase would be sevenfold. It is remarkable that such an increase was not associated with a detrimental effect upon the Ca balance. In this respect it is of importance to consider the observation of Nicolaysen [1934] that the output of Ca in the digestive juices of adult man amounts to about 1 g. daily, that is to say, about twice the daily Ca requirement. (This observation is similar to that of Watson [1933] who observed that the salivary P, mainly inorganic, of sheep can amount to as much as 7·7 g. daily, which is several times the daily requirement for growth.) That reabsorption of this secreted Ca is conducted with an extraordinary degree of efficiency is made clear by considering the phosphatase increases. The enormous increase in secretory activity which must have been associated with the increases observed in faecal phosphatase occasioned no losses of Ca from the organism. It is probably correct to assume that the increase in the volume of the secretions would cause some diminution in the concentration of Ca in the secretions, but it is unlikely that the reduction would be severe, since the electrolyte pattern of the secretions bears a relation to that of the blood (as has been shown by Watson for salivary P and blood P in sheep) and there is no record of increased bulk of ingesta reducing blood Ca.

The efficiency with which the secreted Ca is reabsorbed is probably the outcome of the Ca being in ionized form, or at least in forms readily ionizable under conditions existing within the intestine.

Mitchell [1923–24] found that N excretion was increased by 42% when rats receiving a N-free, low fibre diet had access to filter paper and his 'metabolic N' correction is generally accepted in certain types of protein metabolism studies.

Whiteacre *et al.* [1929–30] considered that high fibre diets reduced the N balances in human subjects but, unfortunately, no analyses of the diets for crude fibre were performed. Morgan [1934] reported that an increased fibre intake had a slight detrimental effect on N retention in man. In the case of the rabbit, Sjollema [1923] failed to find any effect of crude fibre on N retention.

The generally observed detrimental effects on N retention of increased fibre intake were found in the present study. The highest retention was observed during the period of lowest fibre intake and the lowest retention occurred during the period of highest fibre intake. During period 3 a higher retention was observed than in period 2; this may have arisen owing to the shortness of the experimental periods and the lack of accustoming intervals between periods. The percentage of N intake excreted in the faeces for the 0, 10, 20 and 30 % fibre periods were: 10·6, 12·3, 15·0 and 14·4. In this case again an unexpectedly high value occurs in period 3, resulting from the aberrant values found for urinary and faecal N on the first day of this period when, apparently, there was poor absorption of N. If the faecal N value for this day is omitted in calculating the percentage of dietary N lost in the faeces the series becomes: 10·6, 12·3, 14·0 and 14·4. Since the faecal N originates only in part from the dietary N, an attempt is made in Table 5 to recalculate the data in such a manner as to reveal any connexion existing between the increases in faecal N excretion consequent on increased fibre intakes and increased phosphate excretions. It is realized that several debatable assumptions are involved in this approach but, in the absence of values for the different forms of N in the faeces, a first approximation is attained.

Table 5. *Relation of 'N-cost' of dietary fibre to the excretion of phosphatase in faeces*

Period	Faecal N mg.	N originating from intake mg.	'N-cost' mg.	Phosphatase increase	Phosphatase increase / 'N-cost'
1	307	—	—	—	—
2	335	288	47	592	13
3	342	259	83	1008	12
4	324	240	84	1276	15

In column 2 are recorded the faecal N averages from Table 3 (except in the case of period 3, where the aberrant value for the first day has been excluded from the calculation of the average). In column 3 is recorded the quantity of N in the faeces that is calculated to have arisen from and on account of the basal diet intake, the computation being based on the period 1 finding that the faecal N is equal to 10·6 % of N intake when no fibre is added to the diet. As stated above, Mitchell found that the 'metabolic-N' bore a close relation to the quantity of diet consumed; this was confirmed by Adolph & Wu [1934], who demonstrated in addition that increased bulk of ingesta did not interfere with corrected protein digestibility unless the material passed through the intestine at a more rapid rate than usual. Neither Adolph & Wu nor ourselves found any evidence of increased rate of transit when filter paper was fed to rats. In column 4 are given the differences between the values in columns 2 and 3; this fraction is considered to be the portion of the faecal N which was lost to the animal as a result of the quantity of fibre ingested and is referred to as the 'N-cost' of the fraction. In column 5 are given the increases in phosphatase excretion over the basal period 1 level. Finally, in the last column are presented the ratios Phosphatase increase: 'N-cost'.

The agreement between the ratios suggests that there is a relation between phosphatase excretion and increased N excretion on higher fibre intakes. Theoretically, it seems probable that the extra N eliminated is partly in the form of epithelial fragments and partly mucoprotein. It is to be expected that such contributions of these forms of N as occur in the lower regions of the small intestine will escape hydrolysis and absorption. It will certainly be true of those originating in the large intestine.

From the results obtained it is indicated that studies of phosphatase excretion may be valuable aids in investigations of secretory activities of the intestinal tract and this probably applies also in the case of other enzymes. In questions of pathological changes of the tract the estimation might be of value. Concerning the latter, the test might be of use in studies of cases of hypersensitivity of the intestinal tract to fibre in human subjects.

SUMMARY

Increasing the fibre content of the diet of rats to levels as high as 30 % had no influence on Ca retention but reduced the N balance.

Large increases were observed in faecal phosphatase excretion when the fibre intake was raised and this was thought to indicate increased secretory activity within the intestines.

The absence of any depressing effect of large increases in the intestinal secretions on the Ca balance was considered to indicate that the reabsorption of secreted Ca is highly efficient.

REFERENCES

Adolph, Wang & Smith (1938). *J. Nutrit.* **16**, 291.

—— & Wu (1934). *J. Nutrit.* **7**, 381.

Ascham (1930–31) *J. Nutrit.* **3**, 411.

Bloom (1930). *J. biol. Chem.* **89**, 221.

Bodansky (1933). *J. biol. Chem.* **101**, 93.

Christiansen (1936). Undersøgelser over Calciumudskillelse. Thesis: Copenhagen.

Cowell (1937). *Biochem. J.* **31**, 848.

Heymann (1930). *Z. Kinderheilk.* **49**, 748, 761.

—— (1931). *Z. Kinderheilk.* **51**, 673.

—— (1933). *Z. Kinderheilk.* **55**, 92.

McCance & Widdowson (1939). *Biochem. J.* **33**, 523.

Mitchell (1923–24). *J. biol. Chem.* **58**, 873.

Morgan (1934). *J. Amer. med. Assoc.* **102**, 995.

Nicolaysen (1934). *Skand. Arch. Physiol.* **69**, Suppl.

Sjollema (1923). *J. biol. Chem.* **57**, 271.

Watson (1933). *Aust. J. exp. Biol. med. Sci.* **11**, 67.

Westerlund (1938, 1). *Lantbrukshogsk.* **5**, 417.

—— (1938, 2). *Skand. Arch. Physiol.* **80**, 403.

Whiteacre, Willard & Blunt (1929–30). *J. Nutrit.* **2**, 187.

4. THE COMPONENT ACIDS OF PHOSPHATIDES PRESENT IN COW'S MILK FAT

By THOMAS PERCY HILDITCH and LIONEL MADDISON

From the Department of Industrial Chemistry, University of Liverpool

(*Received 14 November 1940*)

Although until quite recently phosphatides were considered to play an important part in the production of the characteristic glycerides present in milk, or even to be the precursors of the latter, a quantitative statement of the composition of the mixed fatty acids in milk fat phosphatides was lacking until the fatty acids of phosphatides from butter milk powder were examined by Kurtz *et al.* [1934]. Previously, however, Osborne & Wakeman [1915] had reported the presence of saturated acids of M.P. 62–65° and of liquid acids of iodine value 85·7 in phosphatides isolated from cow's milk, whilst Sasaki & Hiratzuka [1931], who had prepared purified phosphatides from dried milk, observed lauric, myristic and palmitic acids amongst the fatty acids present, but found no evidence of the presence of unsaturated acids. Kurtz *et al.* [1934] extracted the phospholipids from 300 lb. of buttermilk powder with a mixture of 75 % ethyl alcohol and 25 % of light petroleum and separated the material precipitated from acetone into two fractions, (*a*) phosphatides soluble in ether at 0 and (*b*) substances insoluble in this medium which appeared to be of the nature of sphingomyelin and cerebrosides (*vide infra*, pp. 27, 29). The phosphatides of fraction (*a*) yielded on hydrolysis 62 % of fatty acids, the component acids of which were observed to be myristic 5·2, stearic 16·1, arachidic 1·8, oleic 70·6 and dicostetraenoic 6·3 % (wt.). These authors were unable to detect any palmitic acid, but identified myristic, stearic and arachidic acids in the ester fractions which they obtained; of unsaturated acids, they established the presence of oleic acid in quantity, and also of a highly unsaturated acid of higher molecular weight for which the analytical data indicated a formula $C_{22}H_{36}O_2$, but found no evidence for di- or tri-enoic C_{18} acids. They also found that, in contrast to butter fat, the phosphatides contained none of the lower saturated fatty acids.

We have recently examined the acids from two specimens of milk fat phosphatides, but the quantities of each in our possession did not permit us to separate the 'lecithin-kephalin' fraction from other compounds also present, possibly of the types noted by Kurtz *et al.* [1934] or possibly also including a certain proportion of wax-esters. We have, however, submitted the mixed acids from each specimen to analysis by ester fractionation and have confirmed the findings of other workers that the typical lower saturated acids of cow's milk fat are wholly absent. Our data, like those of Kurtz *et al.* [1934], disclose the presence of up to 10 % of highly unsaturated acids of molecular weight corresponding to members of the C_{20} and C_{22} series (in common with all animal phosphatide component acids so far studied). Our experience differs, however, from that of Kurtz *et al.* [1934] in that, amongst the saturated acids, we observed the presence of relatively large proportions of palmitic acid.

EXPERIMENTAL

The first specimen of milk fat phosphatides which we studied was kindly placed at our disposal by Dr B. Rewald, who had isolated it from non-glyceridic residues obtained in the separation of pure butter fat from Swiss butter. These had been extracted, first with light petroleum and then with a mixture of 80 % alcohol and 20 % benzene, the extract from this second operation being subsequently re-extracted with light petroleum. The combined light petroleum extracts were boiled with acetone and the portion which separated in the cold was the subject of the present work (the material left in solution in acetone was found to be mainly butter fat). In consequence of the differences between the results we obtained with this specimen and those of Kurtz *et al.* [1934] we proceeded to isolate, employing the procedure of Rewald [1937], the corresponding phosphatide fraction from residues from English butter fat clarification kindly provided for us by the Pure Butter Fat Process Co.

Component acids of the phosphatides from Swiss butter residues

The material received from Dr Rewald consisted of friable, dark brown, somewhat hygroscopic granules: it had a saponification equivalent of 221·4 and gave on hydrolysis mixed fatty acids which had (after removal of unsaponifiable matter) sap. equiv. 290·2, I.V. 37·0.

The corresponding material left in solution in acetone in the later stages of Dr Rewald's isolation of the butter phosphatides had sap. equiv. 243·5, I.V. 38·4. We submitted a portion (111 g.) to complete analysis by the usual fractionation procedure and found its component acids to be butyric 3·7, *n*-hexanoic 1·8, *n*-octanoic 1·2, *n*-decanoic 2·4, lauric 2·6, myristic 11·8, palmitic 28·6, stearic 8·9, arachidic 1·2 %: decenoic 0·2, dodecenoic 0·4, tetradecenoic 1·0, hexadecenoic 2·8, oleic 27·3, octadecadienoic 5·8, unsaturated C_{20-22} 0·3 % (wt.). It thus evidently consisted of typical butter fat glycerides.

The Swiss butter phosphatides (40·7 g.) were hydrolysed, first with 0·5 % alcoholic H_2SO_4 (500 ml.) and subsequently with alcoholic KOH. After thorough removal of alcohol from the soaps produced, the latter were dissolved in water, acidified with H_2SO_4 and steam-distilled for 5 hr. The steam-distillate was exhaustively extracted with ether and the ether-extracted aqueous portion (A) and the ether distilled from the extract (E) were examined for acidity. The material extracted by ether (0·57 g., equivalent 229·3) was solid at room temperature; no odour of butyric or other fatty acids of low molecular weight was, moreover, perceptible in the original aqueous condensate from the steam distillation. Both the aqueous portion (A) and the recovered ether (E) nevertheless possessed definite acidity, but this was shown to be due mainly to HCl and, to a less degree, to traces of sulphurous and/or sulphuric acid. Aliquot parts of (A) and (E) were exactly neutralized, evaporated to a small bulk, and any chloride estimated as silver chloride; another aliquot portion of (A) was treated with H_2O_2 in the cold, then concentrated to a small volume, and any H_2SO_4 was determined as $BaSO_4$; a control experiment carried out *ab initio* with the alcohol used in the hydrolysis of the phosphatides served to account for any traces of volatile acids emanating from the alcohol. The results of these determinations are summarized on the following page.

The observed acidity was therefore mainly due to HCl, presumably from NaCl carried down with the phosphatides in the course of their extraction.

Butyric or other low molecular weight acids, present in butter glycerides, were totally absent from the phosphatides.

		Equivalents of acid present
(A) Aqueous steam-distillate after extraction with ether:		
Total acidity by titration		0·02482
Volatile acids from alcohol (control experiment)	0·00023	
Acidity due to hydrochloric acid	0·02184	
Acidity due to sulphurous or sulphuric acid	0·00263	
Total accounted for		0·02470
(E) Ether from ether-extract of steam-distillates:		
Total acidity by titration		0·00098
Acidity due to hydrochloric acid		0·00032

The acids non-volatile in steam were recovered and submitted to lead salt separation. The total fatty matter accounted for was as follows:

	g.	%
Steam-volatile fatty acids	0·57	3·2
Non-steam-volatile fatty acids:		
'Solid' acids S	8·32	46·2
'Liquid' acids L	8·63	48·0
Unsaponifiable matter	0·47	2·6
	17·99	

The yield of fatty acids from the phosphatides (44 %) was low, in consequence of the fact that the small amount of material available precluded further purification of the 'lecithin-kephalin' fraction.

The 'solid' and 'liquid' acids were separately converted into methyl esters and the latter distilled *in vacuo* through an electrically heated and packed fractionating column; the fractions listed in Table 1 were obtained.

Table 1

No.	g.	B.P./0·1 mm.	Sap. equiv.	I.V.
		(i) Methyl esters of 'solid' acids S		
S1	2·53	80–100°	270·7	4·2
S2	1·86	100°	290·2	12·8
S3*	2·40	Residue in column	318·7	—
S4*	1·40	Residue in distilling-bulb	368·9	—
	8·19			
		(ii) Methyl esters of 'liquid' acids L		
L1	2·42	100–120°	279·0	70·6
L2	3·05	120–126°	297·2	103·3
L3	2·28	Residue in column	301·9	109·2
L4*	0·92	Residue in distilling-bulb	448·6	—
	8·67			

* Esters, freed from unsaponifiable matter: S3, sap. equiv. 317·5, I.V. 17·3; S4, sap. equiv. 366·4, I.V. 11·7; L4, sap. equiv. 354·0, I.V. 90·4.

The following acids were identified in certain of the above fractions:

Acids volatile in steam (*0·57 g., equiv. 229·3*). Crystallization from 90 % alcohol gave a crystalline fraction, M.P. 56° (mixed with palmitic acid, M.P. 58–60°); this appeared to be a mixture of palmitic and myristic acids.

'*Solid*' *acids S*. The acids from fraction S1, on crystallization from 90 % alcohol followed by ethyl acetate, gave palmitic acid (M.P. 61·0°, mixed with pure palmitic acid M.P. 62·0°). Those from fraction S3, after four crystallizations from ethyl acetate, yielded pure arachidic acid (M.P., and mixed M.P., 74–74·5°). Those from the residue S4 could not be obtained pure, but gave, from ethyl acetate, a mixture of acids which melted at 76·5° (mixed with pure arachidic acid, M.P. 71–72°); acids of higher molecular weight than arachidic acid were obviously present.

'Liquid' acids L. The acids from fraction L3 were dissolved in anhydrous ether at 0° and treated with bromine; insoluble bromo-additive compounds were precipitated, which commenced to darken at 200–210° and decomposed without completely melting at 224° (characteristic for highly unsaturated C_{20-22} acids).

The presence of arachidic acid is satisfactorily established, but the nature of the saturated acids of higher molecular weight remains uncertain. These may represent wax esters present in traces in the original milk fat and concentrated by the process of extraction in the phosphatide (acetone-insoluble) fraction; or, perhaps more probably in the light of recent work by Kurtz & Holm [1939], they may emanate from bodies of a sphingomyelin or cerebroside nature. In the former case they would be mainly of the formula $C_{26}H_{52}O_2$, in the latter $C_{24}H_{48}O_2$. For the purpose of the present calculations they have been taken as $C_{26}H_{52}O_2$. The final composition of the mixed fatty acids from the Swiss butter phosphatide concentrate is then as in Table 2 (the precision of the analyses is not considered to be of the order attainable with more adequate amounts of experimental material)

Table 2. *Component acids of phosphatides from Swiss butter residues (approx.)*

		Acids non-volatile in steam					
Acid	Steam volatile acids (3·2%)	'Solid' acids S (46·2%)	'Liquid' acids L (48·0%)	Unsaponifiable (2·6%)	Total %	% (wt.)*	% (mol.)*
Myristic	3·03	0·06			3·09	3·2	4·0
Palmitic	0·17	16·20	3·85		20·22	21·0	23·2
Stearic		6·99			6·99	7·3	7·2
As arachidic		11·83			11·83	12·3	11·1
As $C_{26}H_{52}O_2$		4·97			4·97	5·2	3·7
Hexadecenoic			4·13		4·13	4·3	4·8
Oleic		6·03	25·22		31·25	32·5	32·5
As octadecadienoic			6·19		6·19	6·4	6·5
As C_{20-22} unsatd.			7·51		7·51	7·8	7·0
Unsaponifiable		0·12	1·10	2·60	3·82	—	-

* Excluding unsaponifiable matter.

Component acids of the phosphatides from English butter residues

In the hope of obtaining a larger quantity of material for examination, attempts were made to find a suitable source of the milk fat phosphatide fraction in this country. Although, ultimately, a specimen of no larger size than that discussed above was obtained, the results are here included since the fatty acid composition arrived at differed in some details from that of the preceding sample.

In the first place, preliminary experiments on three possible sources of milk fat phosphatides were carried out, namely, on buttermilk powder and on two varieties of residues from butter fat clarification processes. We are indebted to the Wilts United Dairies Ltd. for the buttermilk powder, and to the Pure Butter Fat Process Company who were good enough to supply us with the other two samples. Small quantities of each product were refluxed with ether and the solutions filtered. The matter soluble in ether was recovered and extracted with light petroleum, and the portion passing into solution was recovered and boiled with acetone. The percentages of acetone-insoluble products (i.e. phosphatide concentrates) from the three specimens were:

	Phosphatide concentrate %
Buttermilk powder	0·4
Butter fat residue A	0·7
Butter fat residue B	2·9

The residue A was a thick yellow emulsion of the consistency of cream, whilst residue B was a dark brown semi-solid paste. In consequence of its much higher apparent phosphatide content, the butter fat residue B was selected as the source of larger supplies of phosphatides and about 8 lb. of the material were extracted with ether. The extracted material was treated first with light petroleum and later with a mixture of 80 % of ethyl alcohol and 20 % of benzene, and the crude phosphatides obtained were separated from acetone as previously described. Ultimately, however, only about 42 g. of the purified phosphatides were recovered for further study. (The acetone-soluble matter extracted from the final phosphatide concentrates had sap. equiv. 246·6, I.V. 40·6, and therefore, as in the preceding instance (p. 25), probably consisted mainly of ordinary butter fat glycerides.)

The phosphatide concentrates (38·5 g.) gave on hydrolysis 15·8 g. of fatty acids (with some unsaponifiable matter); the yield of fatty acids was low, as in the case of the Swiss butter phosphatides. After removal of alcohol from the saponified product, followed by acidification with H_2SO_4, no odour of butyric or other low molecular weight acid could be detected, and the distillation of the mixed fatty acids with steam was accordingly omitted. On submission to a lead salt-alcohol separation the mixed acids (15·82 g.) gave 'solid' acids (9·28 g., 58·7 %), 'liquid' acids (5·49 g., 34·7 %) and a small amount of non-fatty material insoluble in ether (1·05 g., 6·6 %). Distillation at 0·1 mm. of the methyl esters of the two groups of acids through the fractionating column gave a series of fractions, and from the analyses of the latter the approximate composition of the mixed fatty acids was found to be as given in Table 3.

Table 3. *Component acids of phosphatides from English butter residues (approx.)*

Acid	'Solid' acids (58·7 %)	'Liquid' acids (34·7 %)	Unsaponifiable (6·6 %)	Total %	% (wt.)*	% (mol.)*
Myristic	4·74	—	—	4·74	5·5	7·0
Palmitic	7·74	3·89	—	11·63	13·4	15·2
Stearic	7·85	—	—	7·85	9·0	9·2
As arachidic	18·18	—	—	18·18	20·9	19·5
As $C_{26}H_{52}O_2$	8·70	—	—	8·70	10·0	7·3
Hexadecenoic	—	4·30	—	4·30	4·9	5·7
As oleic	9·96	10·49	—	20·45	23·5	24·3
As C_{20-22} unsaturated	—	11·11	—	11·11	12·8	11·8
Unsaponifiable	1·53	4·91	6·60	13·04	—	—

* Excluding unsaponifiable matter.

The component acids in this sample of butter phosphatides are of the same general type as those in the Swiss phosphatides, but contain more of the higher saturated acids, i e. a higher proportion of admixed wax esters, or sphingomyelin and cerebrosides, as the case may be. When allowance is made for this, however, it appears that the palmitic and oleic acid contents disclosed by this analysis are somewhat lower than in the preceding case. Acids of the C_{18} group more unsaturated than oleic were not definitely detected, but the small amount of methyl esters of 'liquid' acids available for fractionation may well have been responsible for this.

Discussion and summary

The results of the two analyses of cow's milk phosphatide component acids described above fully confirm the findings of previous investigators that the typical milk fat glyceride acids (butyric, etc.) of low molecular weight are wholly absent from the phosphatide acids. It has been demonstrated [Graham *et al.* 1936; Maynard *et al.* 1938] that the blood phosphatides of the cow are not removed by the mammary gland, and that these are not the precursors of milk fat glycerides. It is now equally well established that the component fatty acids of milk fat phosphatides have little in common with those of the milk fat glycerides; on the other hand, they bear more general similarity to those of the phosphatides of the ox liver [Hilditch & Shorland, 1937], as the following comparison indicates:

Table 4. *Component acids of ox liver and cow's milk phosphatides*

Phosphatides of ...	Ox liver [Hilditch & Shorland, 1937]	Cow's milk fat		
		(Present work)		Kurtz *et al.* [1934]
		'Swiss'	'English'	
Acid	% (wt.)	% (wt.)	% (wt.)	% (wt.)
Myristic	1·3	3·2	5·5	5·2
Palmitic	28·2	21·0	13·4	—
Stearic	14·4	7·3	9·0	16·1
As arachidic	0·2	12·3	20·9	1·8
As $C_{26}H_{52}O_2$	—	5·2	10·0	—
Tetradecenoic	0·7	—	—	—
Hexadecenoic	3·8	4·3	4·9	—
Oleic	17·3	32·5	23·5	70·6
As octadecadienoic	14·2	6·4	-	—
As C_{20-22} unsaturated	19·9	7·8	12·8	6·3

So far as our analyses are concerned, the points of resemblance with the liver phosphatide component acids are the presence, as major components, of palmitic and oleic acids, together with notable proportions of stearic and of highly unsaturated acids of the C_{20} and C_{22} series; the chief points of difference are the minor proportions of octadecadienoic acid in the milk fat phosphatides, and the presence of considerable amounts of arachidic acid and of higher saturated acids (reckoned arbitrarily as $C_{26}H_{52}O_2$). The compounds giving rise to most of these higher saturated acids were separated, by Kurtz *et al.* [1934], by crystallizing the phosphatide concentrates from ether at 0°. Kurtz & Holm [1939] state that the ether-insoluble fraction contains about 80 % of lignoceric acid, the remaining 20 % being a very complex mixture of saturated, unsaturated, hydroxy-saturated and presumably hydroxy-unsaturated acids, the carbohydrate, phosphorus and nitrogen contents of this fraction supporting the view that it is a mixture of sphingomyelin and cerebrosides. It is possible that simultaneous separation from ether of some of the more saturated 'lecithin-kephalin' phosphatides is responsible for the large proportions of oleic acid present in the ether-soluble fraction the composition of which was given by Kurtz *et al.* [1934]; but their failure to detect any palmitic acid in either group of the phosphatides renders the composition of the material which they examined unusual, if not unique, in the records of component acids of phosphatides from any source.

REFERENCES

Graham, Jones & Kay (1936). *Proc. roy. Soc.* B, **120**, 330.
Hilditch & Shorland (1937). *Biochem. J.* **31**, 1499.
Kurtz & Holm (1939). *J. Dairy Sci.* **22**, 1011.
—— Jamieson & Holm (1934). *J. biol. Chem.* **106**, 717.
Maynard, McCay, Ellis, Hodson & Davis (1938). *Mem. Cornell Univ. agric. Exp. Sta.* no. 211.
Osborne & Wakeman (1915). *J. biol. Chem.* **21**, 539.
Rewald (1937). *Le Lait*, **17**, 225.
Sasaki & Hiratzuka (1931). *Proc. imp. Acad. Japan*, **7**, 99.

5. PALM OIL CAROTENOIDS

1. THE ISOLATION OF LIPOID PIGMENTS FROM A 'SHERBRO' PALM OIL

By ROBERT FERGUS HUNTER AND ALAN DALTON SCOTT

From the Central Technical Department, Lever Brothers and Unilever, Ltd., Port Sunlight

(*Received 25 November 1940*)

THE colour of red African palm oils is generally attributed in the main to the presence of carotene, a relatively high proportion of which is assumed to occur in the form of the optically active α-isomer [Kuhn & Brockmann, 1931]. Actually, qualitative chromatographic examination of the unsaponifiable matter of typical palm oils provides immediate evidence of the multiplicity of the coloured constituents exhibiting the usual carotenoid characteristics. It is to be expected that the nature of the carotenoids present in palm oils will vary to some extent with the district of their origin and their previous history. It therefore appeared to us that an examination of some typical palm oils might prove of interest from the point of view of identifying known carotenoids and throwing new light on the problem of their biogenesis; moreover, such investigations might furnish examples of other members of the group which have not previously been detected in nature. In this paper, which is to be regarded as of an introductory character, attention has been directed to an investigation of the carotenoids present in 'Sherbro' palm oil.

With the object of minimizing as far as possible any tendency for the carotenoids to undergo isomeric changes of the type recently described by Zechmeister & Tuzson [1938], and by Carter & Gillam [1939], the saponification of the oil was accomplished at as low a temperature as reasonably possible. Extraction with light petroleum and thereafter with methylated ether, followed by evaporation under reduced pressure in an atmosphere of CO_2, furnished fractions which may be described for the sake of convenience as petrol-soluble unsaponifiable matter and ether-soluble unsaponifiable matter. The former of these, on chromatographic adsorption on a Tswett column of alumina, using a modified technique in which the separation is conducted in an atmosphere of CO_2, gave a series of coloured zones which on elution and subsequent purification furnished beautifully crystalline specimens of α- and β-carotenes and carotenoids having the spectroscopic and other characteristics of γ-carotene and lycopene.

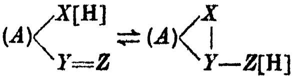
The physical properties of the specimens of α- and β-carotene so obtained indicate that these are almost certainly purer than any which have previously been described in the literature. The dextrorotation of α-carotene in benzene for the sodium D line is recorded for the first time. Curiously enough, nearly all previous measurements have been made with respect to the less intense cadmium line, for which the value $[\alpha]_{6435} = +385°$ has been generally accepted. The value for the shorter wave-length of the sodium D line corresponding to this cannot be deduced in the absence of an accurate knowledge of the rotatory dispersion of the compound, but the rotation of +584° for the helium line 5876, recorded by Smith & Milner [1934] for a specimen melting some degrees lower than ours, is reasonably close to our determination of +640° for the sodium line 5893. Solutions of our specimens of α- and β-carotene in arachis oil which were submitted to Dr H. Wilkinson of our Nutrition Laboratory for biological test provided striking confirmation of Kuhn & Brockmann's [1933, 2] 1:2 ratio for the potencies of α- and β-carotene.

The lycopene zone of the chromatogram of the petrol-soluble unsaponifiable matter, on being eluted and rechromatographed on alumina, furnished upper and lower dark red zones containing lycopene and *neo*lycopene respectively. The closeness of the absorption maxima of *neo*lycopene and γ-carotene would at first sight appear confusing, were it not for the finer differences in adsorptive power which permit the separation of the carotene from the 'total lycopene' zone.

The γ-carotene zone of the original chromatogram, on elution and readsorption on alumina, furnished a single main dark red zone containing γ-carotene. A lighter coloured fringe appeared below this on the second chromatogram, which gave a small quantity of an unidentified carotenoid having maxima of a considerably shorter wave-length than those of γ-carotene. In a subsequent experiment in which more thorough development of the chromatogram of the petrol-soluble unsaponifiable matter was obtained, the zone containing this new carotenoid appeared between γ- and β-carotene. What appears to be *neo*-γ-carotene was obtained when a chromatogram from a concentrate of γ-carotene was very thoroughly developed with a mixture of benzene and light petroleum.

Evaporation of the percolate from the original chromatogram of the petrol-soluble unsaponifiable matter gave a small amount of hentriacontane, accompanied by a quantity of a high-boiling point yellow oil which has not been identified. It may be noted that Carter *et al.* [1939] observed the presence of what appears to be a similar material which was assumed to be of a terpene character, in algae. The ether-soluble unsaponifiable matter on being dissolved in petrol and partitioned with 90% methyl alcohol gave a hypophasic fraction which on chromatographic adsorption on alumina gave a coloured zone containing *neo*lutein. The epiphasic fraction, which contained quantities of sterols, gave on chromatographic analysis three zones containing substances which showed vague but intense spectroscopic absorption. They gave the usual reaction with chloroformic antimony trichloride with sharply defined maxima in the 590 mμ region and were most probably oxidized carotenoids.

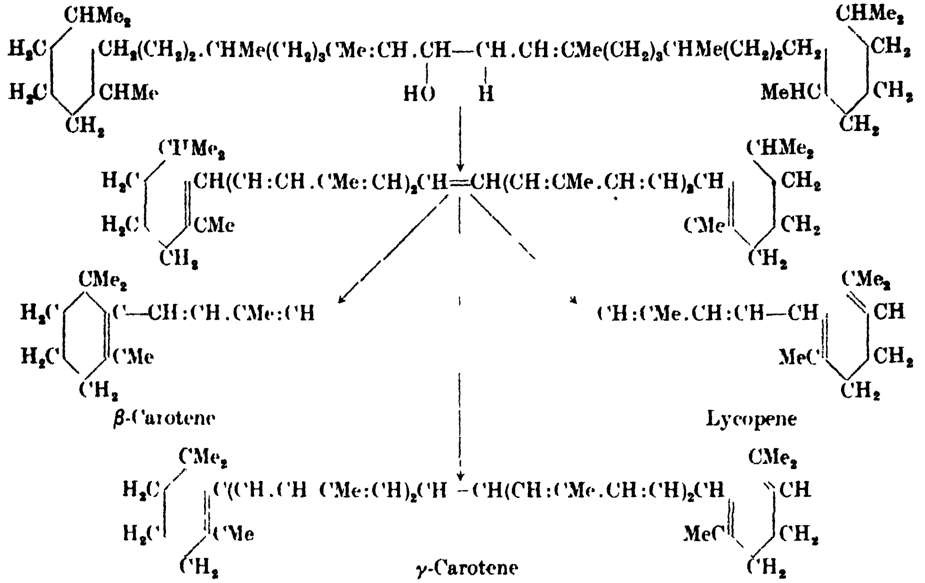
The presence of γ-carotene and lycopene in addition to α- and β-carotenes in this palm oil seems suggestive from the point of view of the biogenesis of these pigments. The photosynthesis of lycopene by dehydrogenation of the product of the benzoin condensation of two molecules of the aldehyde corresponding to phytol has already been suggested by Karrer *et al.* [1930]. That lycopene is the precursor of β-carotene seems to us, however, unlikely, since a ring-chain isomerization of this type finds no parallel in synthetic organic chemistry. If ring-chain prototropy were possible, the presence of the terminal *gem*-dimethyl group of lycopene would, on the basis of the modified strain theory of Ingold and Thorpe, be expected to promote five- rather than six-membered ring formation [cf. Ingold, 1921]. Application of the general expression for ring-chain prototropy



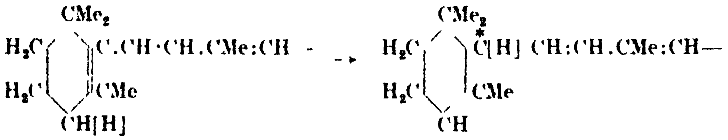
$$(A)\Big\langle\begin{array}{l}X[\mathrm{H}]\\ Y{=}Z\end{array} \rightleftharpoons (A)\Big\langle\begin{array}{l}X\\ |\\ Y{-}Z[\mathrm{H}]\end{array}$$

to lycopene would lead to a cyclic isomeride quite different from β-carotene.

It seems more likely therefore that lycopene, β-carotene and also γ-carotene are formed simultaneously from the same intermediate complex, the γ-isomer representing the cyclization of only one end of the hydrocarbon chain:

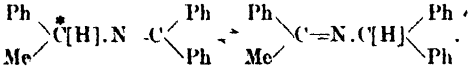


The appearance of α-carotene may be explained as being due to asymmetric isomerization of part of the β-carotene. Such a change would be of the true three-carbon type:

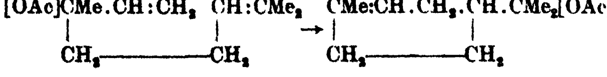


[H] mobile hydrogen atom.

analogous to the racemization of *d*-benzhydrylidene-α-phenylethylamine [Hsu *et al.* 1935]:



The latter change is concerned with the disappearance of optical activity due to mobility of the azomethine triad system $[H]C.N:C \rightleftharpoons C:N.C[H]$, which is closely related to the three-carbon system [Ingold & Shoppee, 1929]. The corresponding process of asymmetric synthesis evidently occurs in biochemical reactions in view of the frequent appearance of optical activity in natural products. A case of isomeric change involving movement of the centre of asymmetry without loss of optical activity, which is of some interest in this connexion, is furnished by the conversion of linalyl acetate into the correspondingly optically active α-terpinyl ester [Stefan, 1898; Zeitschel, 1906; Ingold & Wilson, 1933]:



The isomeric change in this case is, however, of the anionotropic type and differs from the examples studied by Hsu *et al.* [1935] in that the migrating centre of asymmetry does not follow the route of the mobile group.

EXPERIMENTAL

The sample of 'Sherbro' palm oil used for the following experiments was of recent importation and of good colour. The free fatty acid content determined by titration was 15 %, calculated as palmitic acid.

The oil (1500 g.) was saponified in two batches as follows. A solution of KOH (225 g.) in alcohol (95 %; 1300 ml.) was cooled to 35° and the palm oil (750 g.) was gradually added with shaking. After a short time sufficient soap was formed to render the liquids miscible and light petroleum (750 ml.) and water (400 ml.) were added to the mixture, which was heated to boiling and thereafter kept overnight at laboratory temperature. The mixture was heated under reflux for 1 hr. (*ca.* 45°), cooled, diluted with alcohol (95 %; 3500 ml.) and then with water to produce a final dilution to 15 l. The resulting soap solution was thrice extracted with light petroleum and the united extracts were washed with water until free from soap and alkali and dried over anhydrous Na_2SO_4. Evaporation under reduced pressure in an atmosphere of CO_2 in an all-glass Quickfit apparatus furnished the petrol-soluble unsaponifiable matter which amounted to 5·3 g. This procedure, which was used in all evaporations to dryness described in this paper, enables the temperature to be kept below 50°.

The residual soap solution was further diluted and thrice extracted with methylated ether, the combined extracts being washed, dried and evaporated in CO_2 as in the previous case. The ether-soluble unsaponifiable matter weighed 4·1 g.

The petrol-soluble unsaponifiable matter was resaponified by heating with a mixture of light petroleum (330 ml.), alcohol (670 ml.), benzene (20 ml.) and NaOH (10 g.) for 4 hr. The cooled mixture was twice extracted with light petroleum and the united extracts were washed, dried and evaporated as before. The residue so obtained was dissolved in light petroleum (*ca.* 400 ml.) containing benzene (10 ml.), and the solution was passed down a Tswett column (18 mm. bore) of active alumina (British Drug Houses Ltd.) under slight CO_2 pressure. The chromatogram was developed with a 10 % solution of benzene in light petroleum when the following zones were obtained in order of decreasing adsorption: dark red brown (medium depth, zone 1), dark red (narrow, zone 2), dark orange red (narrow, zone 3), red orange (broad, zone 4) and very pale yellow (medium depth, zone 5). Each zone was separated and the organic material was eluted by alternate washings with acetone and ether and recovered by evaporation under reduced CO_2 pressure.

Zone 1. The material extracted from this zone amounted to 1·56 g.; a portion of it dissolved in alcohol (95 %) furnished a small quantity of a crystalline digitonide. This confirmed the expected presence of sterol, but the material was not further investigated. An attempt to crystallize the original material from light petroleum-methanol gave a sticky residue which showed broad but vague maxima at 480–485 mμ and 455 mμ in carbon disulphide. It gave the usual reaction with chloroformic antimony trichloride with a maximum at 590 mμ.

Zone 2. The extract from this zone furnished 0·28 g. of a somewhat sticky solid which was dissolved in a small amount of benzene. This solution was diluted with light petroleum to give a mixture containing approximately 10 % of benzene and adsorbed on a column of active alumina (British Drug Houses Ltd.).

The chromatogram was developed with a similar mixture of benzene and light petroleum when four zones were obtained below a little oxidized material: dark red (broad, zone 2*a*), very dark red (narrow, zone 2*b*), extremely dark red (medium depth, zone 2*c*) and very dark red (narrow, zone 2*d*).

Zone 2a (*lycopene*). The material extracted from this zone weighed 0·15 g. and showed definite maxima at 544 and 505 mμ and a vague maximum at 481 mμ in carbon disulphide. It gave a reaction with antimony trichloride with a maximum at 582 mμ. From a benzene-methanol solution of this a few mg. of a red solid were obtained (M.P. *ca.* 147°) which showed maxima at 545, 507 and 474–475 mμ in carbon disulphide and a maximum at 595 mμ in the antimony trichloride reaction. It appears likely therefore that the displacement of the maximum to 582 mμ in the antimony trichloride reaction with the crude material was due to some colourless impurity which does not affect the maxima of the direct colour spectrum.

Zone 2b (*lycopene, traces of* neo*lycopene*). The residue (0·05 g.) obtained from this could not be crystallized and showed maxima at 543–544, 505 and 478 mμ (vague). It gave a broad maximum at 595 mμ in the antimony trichloride reaction.

Zone 2c (neo*lycopene*). Evaporation of the extract furnished a red gum without any trace of crystalline material which showed maxima at 535, 496 and 469 mμ (vague) in carbon disulphide. This material gave a broad maximum at 595 mμ in the antimony trichloride reaction.

Zone 2d (*γ-carotene*). Evaporation of the extract from this zone gave 0·015 g. of material. No crystals were obtained but portions of the residue showed maxima at 530, 495 and 466 mμ (vague) in carbon disulphide. The antimony trichloride reaction gave a broad maximum at 590 mμ.

Zone 3 (*γ-carotene*). This zone furnished 0·2 g. of a somewhat sticky solid which was dissolved in a small amount of benzene; the solution was diluted with light petroleum and subjected to chromatographic separation on alumina. The chromatogram was developed with a 10 % solution of benzene in light petroleum and formed a single main zone with a lighter-coloured fringe at the bottom. Elution of the main zone gave gummy material which showed bands at 529, 496 and 463 mμ in carbon disulphide and a maximum at 595 mμ in the antimony trichloride reaction. A solution of this in benzene-methanol gave a trace of solid which showed maxima at 530, 496 and 463 mμ in carbon disulphide and a maximum at 595 mμ in the antimony trichloride reaction. Kuhn & Brockmann [1933, 2] record 533·5 mμ for the first maximum of γ-carotene in carbon disulphide, but Carter *et al.* [1939] have drawn attention to the fact that the first maximum observed in a prism spectrophotometer tends to be situated some few mμ further towards the shorter wave-length region than that seen with grating instruments.

Elution of the lower yellow fringe of the chromatogram gave a trace of material which showed bands at 486, 458 and 426 mμ in carbon disulphide and a maximum at 595 mμ in the antimony trichloride reaction. In a later experiment with the same 'Sherbro' palm oil, this substance was observed between the γ-carotene and β-carotene zones in the large chromatogram of the petrol-soluble unsaponifiable matter. In this experiment a γ-carotene concentrate was obtained which was separated by chromatographic analysis into γ-carotene (maxima at 529, 495 and 465 mμ in carbon disulphide and at 502, 473–474 and 449 mμ in chloroform) and what we believe to be *neo*-γ-carotene (maxima at 520, 488 and 455 mμ in carbon disulphide).

Zone 4 (*α- and β-carotenes*). The mixture of carotenes obtained by elution of this large zone was dissolved in benzene (100 ml.). An aliquot portion of this diluted with chloroform gave an $E^{1\%}_{1\,\text{cm.}}$ value of 1710 at 458–459 mμ, indicating a carotene content of about 75 % in the crude material. An alcoholic solution of the latter failed to give a digitonide, suggesting that the colourless impurity was not a sterol.

Portions of the benzene solution corresponding to approximately 0·15 g. of carotene were diluted with light petroleum (9 vol.) and the solutions were passed down Tswett columns of Savory and Moore's 'Mayfair' alumina. The chromatograms were developed with a 25–30 % solution of benzene in light petroleum and the lighter-coloured α-carotene zones were separated from the more strongly adsorbed β-isomer. The pigments were eluted with acetone and the crude carotenes were recovered in the usual way.

A solution of the β-carotene concentrate in acetone deposited a crystalline solid which was collected, dissolved in a 20 % solution of benzene in light petroleum and subjected to chromatographic separation on 'Mayfair' alumina for a second time. The central portion of the β-carotene zone was separated and eluted, and the recovered pigment was crystallized as rapidly as possible from acetone to prevent any isomerization. The β-carotene thus obtained formed beautiful glistening purple red plates, M.P. 184·5° (uncorr.; open melting point tube). A solution of the crystals in chloroform had an $E^{1\%}_{1\,\text{cm.}}$ value of 2310 at 463 mμ, which is appreciably higher than the value recorded by Gillam [1935] for β-carotene in this solvent. The antimony trichloride reaction showed a maximum at 592 mμ with an $E^{1\%}_{1\,\text{cm.}}$ value of 423 at the peak of the intensity. It has been observed that the purest specimens of β-carotene which we have obtained in this and other investigations give a rapidly fading maximum in the blue solution obtained in the antimony trichloride reaction.

A solution of the α-carotene concentrate in acetone furnished a crystalline solid which was dissolved in benzene and the solution was diluted with light petroleum and passed down a column of 'Mayfair' alumina. The chromatogram was developed with 30 % benzene in light petroleum and the main zone of α-carotene was eluted. The α-carotene recovered in the usual way was crystallized from acetone within 1½ hr., when it was obtained in glistening red-brown crystals, M.P. 187–187·5° (uncorr.; open melting point tube). This specimen showed maxima at 511, 479 and 450 mμ in carbon disulphide, at 489, 458 and 430 mμ in chloroform, and at 489, 458 and 431 mμ in benzene. The antimony trichloride reaction showed a main band at 495 mμ, a weak band at 650 mμ, an inflexion at 527 mμ and a weak inflexion at 605 mμ. The $E^{1\%}_{1\,\text{cm.}}$ value for the maximum at 458 mμ in chloroform was 2430.

The optical rotation was determined in benzene solution, using a Hilger polarimeter M. 323 and a G.E.C. Osira sodium lamp as a source of monochromatic light. The instrument was adjusted for a half-shadow angle of 4°. Two determinations, adopting in each case the mean of ten readings, gave the following results:

$$[\alpha]_D = \frac{0{\cdot}162 \times 100^\circ}{2 \times 0{\cdot}0127} = +638^\circ,$$

$$[\alpha]_D = \frac{0{\cdot}276 \times 100^\circ}{2 \times 0{\cdot}02118} = +651^\circ.$$

A similar G.E.C. Osira cadmium lamp gave totally inadequate illumination for use with any carotene solution of sufficient concentration to possess detectable rotation.

Zone 5. Elution of this gave a yellow oil (1·6 g.). A solution of this in alcohol (95 %) gave no precipitate with digitonin. An acetone-light petroleum solution of the oil furnished a small quantity of hentriacontane, which separated from acetone as a waxy white solid, M.P. 64–65°.

The ether-soluble unsaponifiable matter from the original saponification was dissolved in light petroleum (250 ml.) and the solution was extracted ten times with 90 % methyl alcohol (total of 500 ml.). The petrol solution was dried over anhydrous Na_2SO_4 and evaporated to dryness at a temperature below 50°; the residue was adsorbed on a column of 'Birlec' alumina. The chromatogram developed by a 15 % solution of benzene in light petroleum formed three zones: dark red (broad; zone 6), orange red (medium depth; zone 7) and yellow ochre (narrow; zone 8).

Zone 6. An acetone extract from this furnished crystalline sterols and an alcoholic solution of these gave a copious precipitate of digitonides, neither of which were investigated. The colouring matter present showed a broad and vague maximum at about 485–490 mμ in carbon disulphide and gave the usual reaction with chloroformic antimony trichloride with a maximum at 590 mμ.

Zone 7. This furnished a gummy material which showed a similar vague maximum in the 485 mμ region and gave a band at 588 mμ in the antimony trichloride reaction.

Zone 8. This also failed to furnish a crystalline pigment and gave a gum which showed maxima at 505 and 480 mμ in carbon disulphide. The antimony trichloride reaction showed a transient band at 590 mμ, a weak but persistent band at 495 mμ and a slight maximum at 650 mμ which increased during observation.

The hypophasic portion of the ether-soluble unsaponifiable matter recovered from 90 % methyl alcohol was subjected to chromatographic adsorption on a column of 'Birlec' alumina. The chromatogram, although developed with a 35 % solution of benzene in light petroleum, furnished only a single orange red zone (medium depth; zone 9).

Zone 9 (neolutein). The crude carotenoid obtained from this zone formed a gum which gave a negative reaction with alcoholic digitonin. It showed maxima at 501 and 473 mμ in carbon disulphide and a band at 595 mμ in the antimony trichloride reaction.

SUMMARY

1. Examination of the unsaponifiable matter of 'Sherbro' palm oil revealed the presence of α-, β- and γ-carotenes, accompanied by lycopene, *neo*lycopene, *neo*lutein and what appears to be *neo*-γ-carotene. A new carotenoid which is adsorbed at a position intermediate between γ- and β-carotenes on the chromatogram was also observed.

2. The physical properties of the specimens of α- and β-carotenes so obtained indicate that these are probably somewhat purer than any which have been described in the literature.

3. The biogenesis of carotenoids is discussed in the light of the phenomena of ring-chain tautomerism and prototropic change involving asymmetric isomerization. It is suggested that lycopene, β-carotene and also γ-carotene are formed concurrently from the same intermediate complex and that α-carotene may arise from subsequent isomerization of the β-compound.

We wish to thank the directors of Lever Brothers and Unilever Ltd. for permission to publish these results. We also wish to express our thanks to Dr J. R. Edisbury for his help in connexion with some of the spectroscopic measurements.

REFERENCES

Carter & Gillam (1939). *Biochem. J.* **33**, 1325.
—— Heilbron & Lythgoe (1939). *Proc. roy. Soc.* B, **128**, 82.
Gillam (1935). *Biochem. J.* **29**, 1831.
Hsu, Ingold & Wilson (1935). *J. chem. Soc.* p. 1778.
Ingold (1921). *J. chem. Soc.* **119**, 305, 951.
—— & Shoppee (1929). *J. chem. Soc.* p. 1199.
—— & Wilson (1933). *J. chem. Soc.* p. 1493.
Karrer, Helfenstein, Wehrli & Wettstein (1930). *Helv. chim. Acta*, **13**, 1084.
Kuhn & Brockmann (1931). *Hoppe-Seyl. Z.* **200**, 255.
—— —— (1933, 1). *Klin. Wschr.* **12**, 972.
—— —— (1933, 2). *Ber. dtsch. chem. Ges.* **66**, 407.
Smith & Milner (1934). *J. biol. Chem.* **104**, 437.
Stefan (1898). *J. prakt. Chem.* **58**, 111.
Zechmeister & Tuzson (1938). *Biochem. J.* **32**, 1305.
—— —— (1939). *Ber. dtsch. chem. Ges.* **72**, 1340.
Zeitschel (1906). *Ber. dtsch. chem. Ges.* **39**, 1780.

6. THE ANALYSIS OF BLOOD PHOSPHOLIPINS

By W. N. M. RAMSAY and C. P. STEWART

From the Department of Physiology, Royal (Dick) Veterinary College, Edinburgh, and the Clinical Laboratory, Royal Infirmary, Edinburgh

(*Received 4 December 1940*)

While the importance of phospholipins in the metabolism of fats is universally recognized, the most perfunctory examination of the relevant literature is sufficient to show that the methods of analysis at present available are inadequate. Though it is possible to determine with ease and accuracy the total phospholipin content of any biological specimen, an estimate of the partition of the lipoid P among the various phospholipins is by no means as simple. Few workers have even attempted such analyses, and none of the methods proposed is completely satisfactory. Thannhauser *et al.* [1939] have determined sphingomyelin by precipitation of the whole molecule with ammonium reineckate by means of a technique published in an earlier series of papers from the same school. Hydrolysis of the lipin solution with baryta is the preliminary step in their choline estimation, and the concentration of lecithin is estimated from the difference between total choline and total lipoid P. The principal drawback to this method is the amount of material required if the sphingomyelin precipitation is to be really satisfactory. On a small scale it seems that precipitation is frequently incomplete.

A method requiring only very small amounts of material has been published by Kirk [1938]. It is based fundamentally on the difference in the solubilities of the various phospholipins in different solvents. While this principle is perfectly satisfactory as long as its application is restricted to qualitative work on a large scale, it breaks down when applied to the quantitative analysis of phospholipins amounting altogether to hardly more than a milligram. This, however, is apparently not the only fault in Kirk's technique, because his total phospholipin values are far more variable than those generally accepted, and many of them are exceedingly low. In one case, for example, he reports a total phospholipin concentration in red blood corpuscles of 26 mg. per 100 ml. This corresponds to a lipoid P value of about 1 mg. per 100 ml., whereas the normal limits of 10–14 mg. per 100 ml. have rarely been passed in either direction by other analysts. Finally, it may be remarked that Kirk appears to have found little difference between the concentration of phospholipins in erythrocytes and in plasma, whereas it is well known that it is generally greater in the former.

Even before the appearance of these papers we had been engaged on this problem for some time, and it had seemed to us that it was vain to hope for a solution based on solubility differences, especially when working with the small samples which we were proposing to use. Our attention, therefore, was directed towards the possibility of determining the various characteristic residues of phospholipin molecules, such as choline, glycerol and β-aminoethanol, with a view to calculating from them the concentrations of the phospholipins. It is readily seen from Table 1 that the calculation of lecithin, kephalin and sphingomyelin is possible from choline, glycerol and P values, and that β-aminoethanol determinations would afford a very useful check on the other analyses.

Table 1

Substance	Phosphorus	Choline	Glycerol	Colamine
Lecithin	1	1	1	—
Kephalin	1	—	1	1
Sphingomyelin	1	1	—	1

In this paper are described methods for the determination of choline and glycerol, and their application, as indicated above, to specimens of phospholipins prepared from 2 ml. blood: that is to say, about 5–7 mg. mixed phospholipins. Mention is also made of hitherto unsuccessful attempts to evolve a specific micro-method for the determination of β-aminoethanol, and hence of kephalin.

The determination of choline

Of the reagents generally used nowadays for the precipitation of choline we have preferred ammonium reineckate to iodine because the precipitate of choline reineckate is much easier to work with than that of choline enneaiodide. The analysis of the precipitate is also much simpler, although it must be admitted that the application of the reineckate method is limited by the fact that choline reineckate is somewhat more soluble than the enneaiodide.

The method described here is a modification of that of Beattie [1936], whose technique we have found satisfactory except in a few minor details. Our method, however, is very much more sensitive than Beattie's, and can be applied to 1 ml. of a solution containing as little as 0·25 μmol. (0·03 mg.) choline.

Method

Reagents. Barium hydroxide, saturated solution.

H_2SO_4, approximately N and $N/10$.

Ammonium reineckate.

Acetone, ammonia-free, prepared by the distillation of A.R. acetone once from slightly acid solution.

Digestion mixture [Folin & Wu, 1919] diluted with 3 vol. of water.

H_2SO_4, 0·007 N, prepared with indicator according to Conway [1939].

NaOH, 40% and 0·007 N.

Procedure. A volume of lipin solution containing not more than 4·5 μmol. (0·54 mg.) of choline is evaporated to a small volume in a 25 ml. pyrex conical flask with 1 ml. saturated aqueous baryta and the mixture is heated on the steam bath for 1 hr. A further 1 ml. of baryta is then added and the heating is continued for 1 hr. more. The mixture is neutralized to methyl red with H_2SO_4, using first N and then, as the end-point is approached, $N/10$ acid. The solution, with the suspended precipitate of barium sulphate and fatty acids, is evaporated to 0·3–0·5 ml. and transferred to a small filter paper with the aid of a capillary pipette and a rubber teat. The precipitate is washed with successive amounts of hot water until the total volume of filtrate amounts to not more than 1·5 ml. On no account whatever must the filtrate be alkaline, and it should not be more acid than $N/50$. 1 ml. of a cold freshly-prepared saturated aqueous solution of ammonium reineckate is then added and the tube is left in the refrigerator for 20–30 min. to ensure complete precipitation. The pale pink crystalline precipitate of choline reineckate is filtered off through a tiny pad of cotton-wool pressed into the bottom of a small filter-funnel (paper is very difficult to wash free from excess ammonium reineckate) and washed with ice-cold water and a little ether. It is dissolved by pouring a few drops of ammonia-free acetone through the filter, the resulting pink solution being collected in a pyrex test tube. After the addition of a drop of dilute acid to prevent loss of ammonia during the subsequent heating, the acetone is removed by cautious evaporation over a free flame. 0·5 ml. of dilute digestion mixture is then added and the precipitate is ashed. The digest is made strongly alkaline and distilled with steam into 5 ml. 0·007 *N* H_2SO_4. The excess acid is titrated with 0·007 *N* NaOH. The formula of choline reineckate is $HO.C_2H_4.N(CH_3)_3.[Cr(NH_3)_2(CNS)_4]$ and from this it can be seen that 1 mol. of choline yields ultimately 7 mol. of ammonia. Consequently, each ml. 0·007 *N* alkali corresponds to 1 μmol. choline. Table 2 shows that very satisfactory duplicate analyses can be made and that the recovery of added choline is, within the limits of experimental error, quantitative. It is theoretically necessary to do a 'blank' experiment on unhydrolysed lipin extract, but in the case of whole blood we have found the 'blank' to be so very small as to be negligible. This, of course, does not apply to the ammonia-blank of the Kjeldahl determination.

Table 2. *Choline, millimol./100 ml. blood*

Sample	Choline added	Choline found	Recovered Amount	Recovered %
1	—	0·256, 0·257	—	—
2	—	0·217, 0·223	—	—
3	—	0·267, 0·262	—	—
4	—	0·217, 0·217	—	—
5	—	0·268, 0·275	—	—
6		0·220, 0·226	—	—
7	—	0·273, 0·264	—	—
8	—	0·216, 0·218	—	—
8	0·215	0·437	0·220	102
8	0·215	0·429	0·212	99

It remains to discuss the specificity of the method. Ammonium reineckate appears to be a highly specific precipitant for quaternary bases. It has been used for the isolation not only of choline and acetylcholine, but also of neurine, carnitine and sphingomyelin. Lecithin does not, on the other hand, appear to form an insoluble reineckate, and creatinine does not seem to be precipitated until its concentration reaches the quite unphysiologically high level of 5 mg. per ml. Hence, while it is in theory possible that blood might contain appreciable amounts of reineckate-precipitable bases, 'blank' estimations have shown that the amount present is not significant, and in any case it is difficult to imagine any compounds other than the choline-containing phospholipins (and, of course, acetylcholine, which has been shown by Bischoff *et al.* [1931] to be present only in very low concentration) from which reineckate-precipitable base would be liberated by hydrolysis with baryta. Furthermore, we have prepared by the method outlined above a specimen of reineckate from 600 ml. of alcohol-ether extract of blood (equivalent to 22 ml. blood) which, when analysed for nitrogen, gave a value of 23·3 %. The theoretical value for choline reineckate is 23·2 %, but this close agreement does little more than confirm the absence of gross contamination, since six-sevenths of the total nitrogen comes from the reineckate radicle. The crystalline form of the precipitate, however, seemed identical with that of the reineckate prepared from pure choline chloride solution, and it would appear that there might be no great danger in the assumption that choline is actually precipitated as a fairly pure reineckate in these experiments.

The determination of glycerol

The determination of glycerol has never been a simple procedure. On an industrial scale the purity of glycerol is sometimes checked by means of a quantitative chromic acid oxidation. Such a technique is obviously quite unsuitable for the present purpose. More hope is offered by permanganate oxidation. Alkaline permanganate oxidizes glycerol with fair regularity to oxalate, which can then be determined in the usual way by precipitation as the calcium salt. This method has been applied to lipoid glycerol [Dickson, 1935], but has been shown to give somewhat erratic results, largely because of the difficulty of determining oxalate accurately in very low concentration. Fleury & Fatome [1935] have recently published a method which depends on the oxidation of glycerol with an acid solution of sodium or potassium periodate and oxidimetric determination of the excess periodate, in accordance with the following equation:

$$CH_2OH.CHOH.CH_2OH + 2HIO_4 \rightarrow 2HIO_3 + 2HCHO + HCOOH + H_2O.$$

The method as published proved unsuitable for two main reasons: in the first place, it deals with amounts of glycerol which are far too large (5–15 mg.), and in the second, it simply measures the disappearance of an oxidizing agent whose specificity is by no means high. It appeared to us, however, that a solution to the problem might be found in the determination of formaldehyde, since each molecule of glycerol yields two molecules of that compound in this oxidation. While many substances are attacked by periodic acid, comparatively few of these (in general, only primary alcohols in which the primary alcoholic group is directly linked to another partially oxidized carbon atom) yield formaldehyde. The number of these which would be liable to interfere with the present experiments is limited, and the most likely one, glucose, can be readily removed by treatment with copper sulphate and calcium hydroxide. Glycerophosphates would also presumably interfere, but these have been shown by Stewart & Hendry [1935] not to be extracted from blood by the usual alcohol-ether mixture.

Formaldehyde, however, is by no means easy to estimate accurately on a micro-scale, although Schryver [1910] has described an exceedingly delicate and highly specific colour reaction for its detection in minute concentration. Schryver himself claimed that his own technique was satisfactory for quantitative use, but in our hands it has given inconsistent and inaccurate results, owing apparently to side reactions among the various reagents which Schryver employed in rather high concentration. We have consequently found it necessary to modify his technique very considerably, and the glycerol method incorporating the modifications is described below.

Reagents. NaOH, 20% solution.

Copper sulphate cryst., A.R., 10% solution.

Calcium hydroxide, as pure as possible.

Sodium periodate, 0·05 *M* in 3 *N* H_2SO_4 (formula of salt supplied by B.D.H. $Na_5H(IO_5)_2$).

Sodium thiosulphate, 50% solution.

Phenylhydrazine hydrochloride, 'Analar', 0·03 *M* in 0·2 *N* HCl.

Potassium ferricyanide, 'Analar', 0·015 *M*. Neither of these last two solutions should be more than a week old, and both should be kept in the dark.

H_2SO_4, 20% by volume.

Procedure. To a volume of lipin solution expected to contain not less than 0·2 μmol. (about 0·02 mg.) glycerol is added 0·2 ml. 20% NaOH. The solution is made up to about 10 ml. with alcohol and evaporated on the hot-plate until its volume does not exceed 1 ml. This evaporation should take about 45 min., and great care must be taken that at no time does any part of the bottom of the flask become dry, otherwise appreciable quantities of glycerol will be volatilized and lost. The residual fluid is transferred with the aid of a capillary pipette to a narrow centrifuge tube graduated at 3 ml., and the flask is rinsed out with several small volumes of hot water. When the solution has cooled to room temperature, 0·3 ml. 10 % copper sulphate, a knife-point of slaked lime and sufficient water to make up the volume to 3 ml. are added. The tube is left to stand for 1 hr., during which it is frequently shaken, and it is then centrifuged. An aliquot of the supernatant fluid, usually 2·5 ml., is pipetted into a 10 ml. round-bottomed pyrex flask with a neck ground to fit a micro-Kjeldahl head with the long tube removed (see Fig. 1). 1 ml. acid sodium periodate solution is added and the flask left to stand for 4–5 min. (not longer). The excess periodate is reduced to iodide by the dropwise addition of 50 % sodium thiosulphate, of which excess should be avoided. The contents of the flask, after neutralization with 20 % NaOH (3 or 4 drops extra being added), are distilled into 2 ml. acid phenylhydrazine solution. When the flask is almost dry a little water is added and the distillation is repeated to ensure that all the formaldehyde passes over. It is important that the solution should not become acid during the distillation. Methyl red and phenol red are suitable indicators for observing this precaution. The distillate is diluted to about 20 ml. and 2 ml. potassium ferricyanide are added. After 25–30 min., during which a colour varying from pale yellow to a rich dark sherry develops, the solution is diluted in a 50 ml. volumetric flask to about 30 ml., and 10 ml. 20 % H_2SO_4 are added. This changes the colour to a rose-red. The solution is made up to the mark with water and compared with a standard made by oxidizing a measured volume of standard glycerol solution. Since the intensity of the final pink colour is not quite proportional to the concentration of formaldehyde, and hence to the concentration of glycerol, it is important to employ a standard the concentration of which approaches that of the unknown fairly closely. Table 3 gives a few typical results, including not

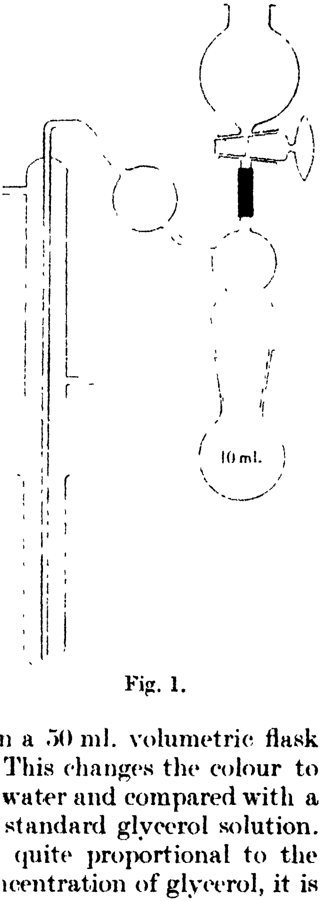


Fig. 1.

Table 3. *Glycerol, millimol./100 ml. blood*

Sample	Added	Found	Recovered	
			Amount	%
1	—	0·088, 0·090	—	—
2	—	0·127, 0·124	—	—
3	—	0·076, 0·0735	—	—
4	—	0·152, 0·147	—	—
5	—	0·178, 0·176	—	—
1	0·1025	0·197	0·108	105
2	0·1025	0·221	0·096	94
2	0·1025	0·224	0·099	97

only duplicate analyses of alcohol-ether extracts of whole blood but also recoveries of added glycerol.

These two methods have been applied, in conjunction with a standard lipoid P technique [Stewart & Hendry, 1935], to specimens of phospholipins prepared from whole blood in the following way.

50 ml. alcohol-ether extract of blood [Bloor, 1928] were evaporated just to dryness on the hot-plate and finally in the steam-oven. The residue was immediately taken up in light petroleum and the turbid solution transferred to a centrifuge tube where it was concentrated to about 0·3 ml. by immersion in a beaker of hot water. Acetone was then added from a 5 ml. pipette, very slowly and with continuous shaking so that the phospholipins separated as a flocculent white precipitate and not as a tarry mass. Precipitation was completed by adding 3 drops of a saturated alcoholic solution of magnesium chloride and allowing the tube to stand for 2 hr., preferably in the refrigerator. At the end of that time any precipitate adhering to the walls of the centrifuge tube was carefully removed by scratching and the precipitate was thrown down in the centrifuge. It invariably settled rapidly and completely and was twice washed at the centrifuge with 5 ml. cold acetone before being taken up in 10 ml. of a mixture of 3 parts of ether, 2 of alcohol and 1 of water, in which it gave a perfectly clear, almost colourless solution. The entire procedure could be carried out in about 3 hr., and during this time the phospholipins spent 2 hr. in the refrigerator, which should have minimized the oxidation by atmospheric oxygen to which these compounds are so very susceptible. Analyses showed that the procedure extracted 92–97 % of the P in the alcohol-ether extract, so that not only is this P virtually all phospholipin-P, but the extraction and precipitation with acetone and alcoholic magnesium chloride are also satisfactory and complete.

Aliquot portions of the ether-alcohol-water solution were analysed for glycerol (2 ml.) and choline (2 ml.) by the methods just described, and for phosphorus (1 ml.) by the method of Stewart & Hendry [1935]. The results of 12 analyses on specimens of whole blood from healthy men in their early twenties are given in Table 4. Table 5 shows the concentrations of lecithin, kephalin and sphingomyelin calculated from the figures in Table 4, the calculations being based on the assumption that these are the only choline-, phosphorus- and glycerol-containing compounds present in the material analysed; this Table also shows the same results calculated as percentages of the total phospholipins.

The figures in Table 5 show one or two rather interesting facts. In the first place, it is clear that although the phospholipins of blood have often been loosely described as 'lecithin', lecithin itself actually accounts for only a small proportion

Table 4

No. of sample	Phosphorus milliatoms/100 ml. blood	Choline millimol./100 ml. blood	Glycerol millimol./100 ml. blood
1	0·432, 0·420	0·274, 0·283	0·178, 0·176
2	0·333	0·290, 0·298	0·147, 0·151
3	0·355	0·300	0·109
4	0·336	0·283	0·124
5	0·340	0·255	0·119
6	0·366	0·271	0·138
7	0·316	0·256	0·111
8	0·319	0·227	0·127
9	0·352	0·275	0·108
10	0·351	0·245	0·104
11	0·347	0·261	0·103
12	0·380	0·292	0·113

Table 5. *Concentrations of phospholipins in blood samples*

	Lecithin		Kephalin		Sphingomyelin	
No. of sample	millimol./100 ml.	% of total	millimol./100 ml.	% of total	millimol./100 ml.	% of total
1	0·028	6	0·148	35	0·250	59
2	0·110	33	0·039	11	0·184	56
3	0·054	15·5	0·055	15·5	0·246	69
4	0·071	23	0·053	14	0·212	63
5	0·034	13	0·085	25	0·211	62
6	0·043	11	0·095	26	0·228	63
7	0·052	16	0·059	19	0·204	65
8	0·035	14	0·092	26	0·192	60
9	0·031	9	0·077	22	0·244	69
10	0·000	0	0·104	30	0·245	70
11	0·017	5	0·086	25	0·244	70
12	0·025	9·5	0·078	20·5	0·267	70
	Mean	13		22		64

of the total. In one case (No. 10), indeed, no lecithin at all was found. While errors in estimations will have been magnified in the calculations, it seems highly improbable that this low concentration of lecithin would be either artificial or fortuitous in every one of 12 consecutive analyses. A second point of interest in these tables is that sphingomyelin is quantitatively by far the most important of the phospholipins present. Thirdly, in nearly all cases the concentration of kephalin is considerably greater than that of lecithin. Even so short a series, moreover, has demonstrated clearly that the proportion of sphingomyelin is fairly constant. It seems possible that this constancy might be emphasized by a series of analyses on plasma and corpuscles separately.

It is difficult to compare these findings with those of Kirk [1938], since, as we have already mentioned, there appear to be grounds for doubting the accuracy of his results. Other workers have, however, recently published comparable figures. Williams *et al.* [1938] have given figures for kephalin concentrations in blood plasma and corpuscles obtained in much the same way as our own by difference between lipoid P and choline. Their results are somewhat higher than we should have expected from our own analyses of whole blood, but this may be explained by the fact that they carried out the precipitation of choline reineckate in a solution sufficiently acid to interfere with the precipitation. This may have led to low results for choline and consequently high kephalin figures. The latter, nevertheless, are of the same order as our own.

Other interesting figures are those of Thannhauser *et al.* [1939] for blood serum and various tissues. These again show a remarkable constancy in sphingomyelin concentration, although it must be admitted that as a rule their results for serum sphingomyelin are rather lower than we should expect from our results with whole blood.

The phosphorus-fatty acid analyses of Stewart & Hendry [1935] indicated that approximately one-half of the total phospholipins of whole blood consisted of sphingomyelin, a proportion somewhat lower than that observed in the present work. Neither series is long, so that the difference may be a chance one. On the other hand, Stewart & Hendry based their admittedly rough conclusion on the ratio of P atoms to fatty acid molecules in the phospholipin precipitate, and a very slight oxidation of the fatty acids before estimation would result in a shift of this ratio sufficient to account for the observed discrepancy. For a mixture containing 50 % sphingomyelin the ratio P:—COOH is 1:1·5; for a mixture containing 64 % it is 1:1·36. Hence, a 10 % increase in the number of carboxyl groups is all that it is necessary to postulate in order to make the two sets of figures agree, and it seems not unreasonable to suggest that such a small amount of oxidation may have taken place. A few preliminary analyses have suggested that on occasion the increase of carboxyl groups may be very much greater than this, and this conclusion is supported by the work of Paal [1929] who studied the oxidation of alcoholic solutions of lecithin and found the number of free carboxyl groups to increase very rapidly.

The attempted estimation of β-aminoethanol and kephalin

During the investigation of substances which might have interfered with the determination of glycerol, it was observed that β-aminoethanol was oxidized slowly in acid and rapidly in alkaline solutions of periodate in the following way:

$$CH_2OH.CH_2NH_2 + IO_4^- \rightarrow IO_3^- + HCHO + H_2O + NH_3.$$

This equation has been verified, not only by the estimation of the periodate used up and the formaldehyde produced, but also by the determination of the ammonia. Several methods were used, depending on the quantities of β-aminoethanol under consideration, and much the most satisfactory proved to be the method of Conway [1939], with which it was practicable to determine accurately the ammonia from as little as 0·2 μmol. β-aminoethanol. The reaction, which appeared to be highly specific, was carried out simply by mixing in the outer chamber of a Conway unit 0·5–1·5 ml. β-aminoethanol solution, 0·5 ml. 0·05 *M* sodium periodate (dissolved in water with the minimum amount of H_2SO_4) and 1 ml. saturated aqueous K_2CO_3. The ammonia formed was allowed to diffuse into standard H_2SO_4 in the inner chamber, the excess acid being titrated after the completion of the diffusion. When the method was applied to hydrolysed lipoid extracts figures which were certainly of the right order were obtained, but it did not seem possible, except on rare occasions, to recover more than 65–75 % of added β-aminoethanol. Various methods were tried for the purification and separation of the base before estimation; these did not alter materially the results of the analysis, but they did not enhance the recovery of added β-aminoethanol to any great extent. A certain amount of success, but not sufficient to justify the use of the method, attended the adsorption of the β-aminoethanol, after hydrolysis of the lipins, from slightly alkaline solution on to frankonit KL, followed by periodate oxidation of the base *in situ* on the adsorbent.

SUMMARY

Methods for the determination of choline and glycerol in lipin solutions are described, with their application to the separate determination of the various phospholipins in whole blood.

In 12 blood samples the concentration of sphingomyelin ranged from 56 to 70 % of the total phospholipin, with a mean of 64 %. In most of the cases the concentration of kephalin appeared to be considerably greater than that of lecithin.

One of us (W. N. M. R.) acknowledges gratefully a Carnegie Research Scholarship during the tenure of which the early part of this work was carried out; some of the expenses of the work were borne by grants from the Earl of Moray Endowment of the University of Edinburgh.

REFERENCES

Beattie (1936). *Biochem. J.* **30**, 1554.
Bischoff, Grab & Kapfhammer (1931). *Hoppe-Seyl. Z.* **199**, 135.
Bloor (1928). *J. biol. Chem.* **77**, 53.
Conway (1939). Micro-diffusion Analysis and Volumetric Error. London.
Dickson (1935). Thesis, Edinburgh.
Fleury & Fatome (1935). *J. Pharm. Chim., Paris*, viii, **21**, 247.
Folin & Wu (1919). *J. biol. Chem.* **38**, 81.
Kirk (1938). *J. biol. Chem.* **123**, 623.
Paal (1929). *Biochem. Z.* **211**, 244.
Schryver (1910). *Proc. roy. Soc.* B, **82**, 226.
Stewart & Hendry (1935). *Biochem. J.* **29**, 1683.
Thannhauser, Benotti & Reinstein (1939). *J. biol. Chem.* **129**, 709.
Williams, Erickson, Avrin, Bernstein & Macy (1938). *J. biol. Chem.* **123**, 111.

7. THE PLASMA-CELL PARTITION OF BLOOD LEAD

BY S. L. TOMPSETT AND A. B. ANDERSON[1]

From the Biochemical Laboratory, Department of Pathology of the University and Royal Infirmary, Glasgow

(*Received 30 December 1940*)

THE distribution of lead between the cells and plasma of blood has been the subject of various reports. According to Aub *et al.* [1926], in experimental lead poisoning 80 % of the lead is in the plasma; Blumberg & Scott [1935] find 90 % in the cells in lead poisoning in man and at least half and usually more in the cells in normal blood; Teisinger [1936] finds that the lead is divided between the cells and plasma in a variable ratio, while Willoughby & Wilkins [1938] could detect no lead in the serum of 90 % of samples of normal blood.

In view of these different findings a further investigation of the plasma-cell partition was undertaken and the results are reported here.

EXPERIMENTAL

For the determination of lead in fresh human blood approximately 40 ml. blood were withdrawn from a vein and mixed with a minimal amount of potassium oxalate Analar; one portion was then centrifuged and the plasma separated. Lead was estimated in the whole blood and plasma by the method described previously [Tompsett & Anderson, 1935; 1939; Tompsett, 1939]. In two cases a determination of cell volume was made by means of the haematocrit and the actual lead concentration in the cells calculated. The results obtained with blood from one normal laboratory worker, two hospital patients with a history of possible exposure to lead but with no obvious poisoning, and three cases of lead poisoning, are given in Table 1. It will be seen that the ratio of whole blood lead to plasma lead is fairly constant, irrespective of the amount of lead present. The concentration in whole fresh blood is approximately three times that in the plasma. Case No. 6 is exceptional in having a ratio of only 1·6 : 1.

A supply of normal stored blood was available in bottles discarded from a blood bank at the end of the period of storage. It was possible to use large volumes of this blood, and 100 ml. of whole blood and 100 ml. of plasma were taken from each bottle for analysis. A correction was made for the dilution due to the sodium citrate and saline anticoagulant solution in the bottles and for the small amount of lead in this solution. The results of the analyses of eight samples, which are given in Table 1, are thus calculated for the original blood, and show a distribution of lead between the cells and plasma similar to that found for fresh blood, but with a slightly higher ratio of lead in whole blood.

Two young male rabbits were given lead by mouth in the form of lead acetate mixed with a diet of crushed oats and bran. They received approximately 30 mg. of lead per kg. per day, the one for 8 and the other for 24 days. Blood films were examined for punctate basophilia at intervals, and at the end of the periods of lead administration the animals were anaesthetized with ether, bled by heart

---

[1] Carnegie Teaching Fellow, University of Glasgow.

Table 1. *Lead concentration in μg. per 100 ml. whole blood, plasma or cells*

Disease	Whole blood	Plasma	Cells	Whole blood / Plasma
1. Normal	69	28	—	2·5
2. Lead poisoning	136	46	—	2·9
3. Lead poisoning	366	118	—	3·1
4. Neuritis	40	15	—	2·7
5. Lead poisoning	128	45	258	2·8
6. Undiagnosed	46	28	73	1·6

Stored normal human blood

Whole blood	Plasma	Whole blood / Plasma
76	27	2·8
24	7	3·4
43	12	3·6
31	9	3·4
48	14	3·4
20	6	3·3
20	5	4·0
58	18	3·2

puncture and killed. The blood was allowed to clot and lead was estimated in the separated serum and clot. Two other young males were each given a single dose of 0·5 g. lead acetate in solution by stomach tube, and blood was taken as before, in the one 20 hr. and in the other 48 hr. after lead administration, and lead was again estimated in the serum and clot. Blood lead was also estimated in a control litter-mate and in two stock female rabbits. The concentration of lead in the whole blood was found by adding the total amount in the serum to the total in the clot and correcting for volume. The results are shown in Table 2. The distribution in normal rabbit blood was of the same order as in blood from human subjects. Rabbits nos. 2 and 3, receiving lead in the food, had a raised blood lead, no. 2, which received lead for 8 days, showing a slight relative increase in the serum lead, while in no. 3, which received lead for 24 days, the serum lead concentration was the same as that of the whole blood. Administration of lead in one large dose by stomach tube caused a greater rise in whole blood lead; in no. 4, 20 hr. after administration of lead, the concentration in the serum was nearly equal to that in the whole blood, while in no. 5 the serum lead concentration was 50 % greater than that of the whole blood.

Table 2. *Rabbit blood: lead concentration in μg. per 100 ml. whole blood or serum*

	Whole blood	Serum	Whole blood / Serum
1. Control	37	17	2·2
2. 30 mg. lead per kg. for 8 days	79	41	1·9
3. 30 mg. lead per kg. for 24 days	85	86	1·0
4. 0·5 g. lead acetate by stomach tube, bled 20 hr. afterwards	102	96	1·0
5. 0·5 g. lead acetate by stomach tube, bled 48 hr. afterwards	112	153	0·7
6. Normal female	11	4	2·75
7. Normal female	53	21	2·5

The blood films of these animals all showed a great increase in polychromatic cells after lead administration, but no. 2 was the only one which had a definite punctate basophilia.

In view of the possibility that lead might be present in the cells as a soluble soap, e.g. lead oleate, a sample of 20 ml. of fresh normal human blood was extracted with alcohol-ether mixture, as in Bloor's method for the extraction of the total lipoids of blood, and then lead was estimated in the extract and residue. No appreciable amount of lead was found in the extract, while the residue contained a normal amount.

Discussion

The accuracy of the analytical method employed is of paramount importance in investigations of this nature, and faults in the methods used may account for some of the widely different distributions of lead in the blood which have been reported. We have criticized some of these methods in a previous paper, in which we first described the method at present in use [Tompsett & Anderson, 1935]. This method consists essentially in the extraction and separation of the lead as an ether-soluble complex with sodium diethyldithiocarbamate and its subsequent estimation colorimetrically with dithizone. Kench [1940] has used this method with slight modifications for the estimation of lead in urine and obtained accurate results. Unfortunately he is wrong in stating: 'Tompsett & Anderson [1935; 1939] have extracted lead from biological materials as a complex either with sodium diethyldithiocarbamate or with dithizone, both substances being stable in the presence of ammoniacal cyanide.' One of the objects of our first paper was to advocate the use of sodium diethyldithiocarbamate instead of dithizone for the extraction of lead. Speaking of the dithizone method we said, 'we have found that in practice, when used to separate lead from solutions of the ash of urine, liver etc., the method gave erratic results'.

The relatively constant ratio between the concentrations of lead in human whole blood and in plasma, which is the same in normal bloods as in those from cases of lead poisoning, suggests that an equilibrium is reached in the distribution between cells and plasma. By contrast, when in the rabbits active absorption of lead was taking place this equilibrium was not reached and lead was present in greater concentration in the plasma. Thus it is possible to reconcile Aub's finding of 80% of lead in plasma in experimental lead poisoning with the later reports that most of the lead is in the cells in clinical lead poisoning.

The form in which lead is present in the blood is undecided. Most of the investigations of the question have been made on serum or blood to which lead salts have been added *in vitro*, or on the blood after intravenous injection of lead salts. These experiments can all be criticized on the grounds that they are unphysiological. The findings reported here do not provide any data as to the nature of blood lead, but exclude the possibility of an ether-soluble form.

Summary

1. The concentration of lead in human whole blood was approximately three times that in the plasma of normal subjects and of patients with lead poisoning.
2. In stored blood the ratio of lead concentration in whole blood to that in plasma was slightly higher than in fresh blood.
3. In rabbits absorbing lead by mouth the concentration of lead in the plasma was equal to or greater than that in whole blood.
4. The lead in normal human blood was not extracted by ether.

REFERENCES

Aub, Fairhall, Minot & Reznikoff (1926). Lead Poisoning, p. 25.
Blumberg & Scott (1935). *Johns Hopk. Hosp. Bull.* **56**, 311.
Kench (1940). *Biochem. J.* **34**, 1245.
Teisinger (1936). *Z. ges. exp. Med.* **98**, 520.
Tompsett (1939). *Biochem. J.* **33**, 1231.
— — & Anderson (1935). *Biochem. J.* **29**, 1851.
—— —— (1939). *Lancet*, p. 559.
Willoughby & Wilkins (1938). *J. biol. Chem.* **124**, 639.

8. STUDIES IN DETOXICATION

8. ON THE ALLEGED FORMATION OF *p*-HYDROXYLAMINOBENZENESULPHONAMIDE AND *p*-AMINOPHENOL FROM SULPHANILAMIDE *IN VIVO*, ON THE COLOUR REACTIONS USED FOR THEIR DETECTION, AND ON THE POSSIBLE FORMATION OF AMINOPHENOLSULPHONAMIDES

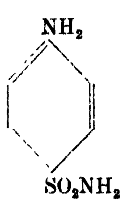
By W. V. THORPE and R. TECWYN WILLIAMS,
and in part JEAN SHELSWELL[1]

From the Physiology Department, Medical School, Hospitals Centre, Birmingham

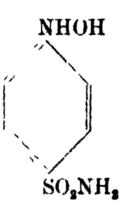
(*Received 19 December 1940*)

When sulphanilamide is adminstered to an animal, it is mainly excreted free and acetylated; a small portion, however, undergoes oxidation, and since the oxidized portion may be responsible for certain toxic effects, its characterization becomes a question of importance. So far reliable identification of the oxidation products of sulphanilamide has not been accomplished, although a few suggestions as to their nature have been offered [e.g. Mayer, 1937; James, 1940; Rimington & Hemmings, 1939; Rosenthal & Bauer, 1939]. The compounds most favoured have been *p*-hydroxylaminobenzenesulphonamide and *p*-aminophenol: claims that these substances appear in urine after ingestion of sulphanilamide have been based mainly on certain colour reactions. It is one object of this paper to show that these reactions are not specific and do not in any way prove the presence of these substances.

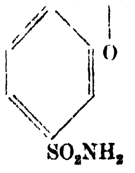
A consideration of what is known about the biological oxidation of various aromatic compounds suggests that oxidation of sulphanilamide (I) *in vivo* might yield the following compounds: *p*-hydroxylaminobenzenesulphonamide (II), 2-oxybenzoxazole-6-sulphonamide (III), 1-amino-2-hydroxybenzene-4-sulphonamide (IV) or 1-amino-3-hydroxybenzene-4-sulphonamide (V).



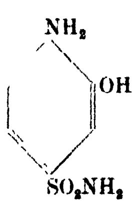
I



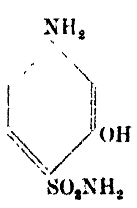
II



III



IV



V

There is the further possibility that dihydric phenols or 4:4′-disulphonamidoazoxybenzene might be formed. The aminophenols IV and V could be excreted in conjugation, the hydroxyl groups with glucuronic acid or sulphuric acid and the amino groups with acetic acid. It is important to remember this when some of the colour tests are applied.

---

[1] Working under Caroline Harrold Research Grant, University of Birmingham.

The stability of the sulphonamide group [cf. Gray *et al.* 1937] makes it improbable that it would be eliminated *in vivo*, so that, on theoretical grounds, the formation of *p*-aminophenol from sulphanilamide is unlikely. We have, nevertheless, examined carefully a number of urines, both human and rabbit, but in no case have we detected *p*-aminophenol. The indophenol reaction which has been frequently used for the detection of *p*-aminophenol can be misleading, and we have devised a modification of the test which is less liable to misinterpretation.

Hydroxylamine derivatives have been postulated as intermediates in the metabolisms of aryl-amino and nitro compounds by several workers [e.g. Ellinger, 1920; Lipschitz, 1920; Böhm, 1939] and some claims to the isolation of such derivatives have been put forward. In 1937 Mayer postulated the slow formation of *p*-hydroxylaminobenzenesulphonamide in the body as the basis for the activity of sulphanilamide. This view has received support from workers who claim to have detected this hydroxylamine in urines of man, dogs, rabbits and mice receiving the drug [e.g. James, 1940; Rosenthal & Bauer, 1939; Rosenthal, 1939, 1]. The claims have been largely based on two colour reactions which, as we shall show, are not specific. We venture to suggest that some of those who support the view that *p*-hydroxylaminobenzenesulphonamide is formed and excreted in the biological oxidation of sulphanilamide have been unduly influenced by the analogy that *β*-phenylhydroxylamine is supposed to be formed during the biological oxidation of aromatic amines such as aniline and acetanilide. Brownlee [1939], Rimington & Hemmings [1939], and James [1940], for example, state that Ellinger [1920] isolated acetylphenylhydroxylamine from the blood of cats poisoned with acetanilide. Now this is not true. Ellinger isolated 1·8 mg. of a substance which he suggested was acetylphenylhydroxylamine 'vorbehaltlich der übereinstimmenden Elementaranalyse'. The analysis was not performed and his other evidence was not conclusive. Again, Lipschitz [1920] claimed that *m*-nitrophenylhydroxylamine was formed from *m*-dinitrobenzene by excised tissues. His evidence consisted of comparative tests unsupported by isolation or any specific test. Undue importance has been given to the power of *β*-arylhydroxylamines to form methaemoglobin in blood. This is a property shared with several substances, including 1-amino-2-hydroxybenzene-4-sulphonamide [Thorpe & Williams, 1941], and is hardly more specific than the possession of reducing properties.

So far as we are aware, conclusive evidence for the formation of an arylhydroxylamine derivative in the mammal has never been produced. In order to justify such a claim, it is essential that the compound, or a derivative, should be isolated in a pure condition, subjected to full elementary analysis, and compared with an authentic synthetic specimen. James's [1940] claim to have isolated *p*-hydroxylaminobenzenesulphonic acid and *p*-acetylhydroxylaminobenzenesulphonamide should be rejected on this account. Further, James states that the latter compound did not melt up to 300°, but Bratton *et al.* [1939] have synthesized the compound and shown that it has M.P. 227–229·5°. This we have confirmed [Thorpe & Williams, 1941].

There is no particular reason why biological oxidation of an aromatic amine should involve the formation of a hydroxylamine. The direct oxidation at some other position in the ring is just as feasible. In the anilines, it is known that biological oxidation takes place in the *ortho* and *para* positions to the —NH_2 [cf. Jaffé & Hilbert, 1888; Horn, 1936, 1, 2; 1937]. In sulphanilamide only the *ortho* or *meta* positions are available. Other work in progress in this laboratory on the metabolism of *p*-hydroxybenzenesulphonamide shows that this sulphonamide undergoes oxidation to a small extent. It gives rise in the rabbit to small amounts of a catechol derivative, the main products being *p*-sulphonamidophenylglucuronide (isolated as Ba salt) and an ethereal sulphate. These considerations led us, *a priori*, to favour 1-amino-2-hydroxybenzene-4-sulphonamide as the oxidation product of sulphanilamide *in vivo*.

The benzoxazolone derivative (III) has been considered since benzoxazolone derivatives have been isolated from urine after feeding animals with certain aromatic amino compounds which lead to the *in vivo* formation of *o*-aminophenols, e.g. formanilide [Kleine, 1896–7], acetanilide and *o*-acetotoluidide [Jaffe & Hilbert, 1888] and salvarsan [Sieberg, 1916]. It is questionable whether these benzoxazolone derivatives were formed *in vivo* or from *o*-aminophenols during the isolation from the urines; this point is now being investigated in this laboratory. We have obtained no evidence for the formation of such a derivative from sulphanilamide. The synthesis of the compound (III) is described in the following paper [Thorpe & Williams, 1941].

In our opinion the most likely oxidation products of sulphanilamide are the aminophenolsulphonamides (IV) and (V). Such compounds would account for the increased excretion of ethereal sulphate after ingestion of sulphanilamide observed by Shelswell & Williams [1940]. We have synthesized both of these compounds. The properties of 1-amino-2-hydroxybenzene-4-sulphonamide are compatible with those of a substance in fractions obtained from urine after feeding rabbits with sulphanilamide. But our experiments on these lines are not yet complete, and we do not claim to have identified this compound. Scudi's [1940] report that he has isolated a hydroxy derivative of sulphapyridine (confirmed by elementary analysis) lends some support to our view that phenolic compounds are formed from sulphanilamide. If the formation of an *o*- or *m*-aminophenolsulphonamide could be proved, it would argue against the correctness of the hydroxylamine hypothesis, in that there is no example in the literature of the transformation of a hydroxylamine into an *o*- or *m*-aminophenol.

The indophenol test for p*-aminophenol*

This reaction has been applied to the detection of *p*-aminophenol in urines after administration of certain drugs, e.g. acetanilide, phenacetin, sulphanilamide. *p*-Aminophenol is oxidized in the presence of phenol to an indophenol dye, which is blue in alkaline and red in acid solution. We found chromic acid [Müller, 1888; Binet, 1889] a less satisfactory oxidizing agent than bleaching powder or calcium hypochlorite [Herrick & Irons, 1906].

Our interest in this test arose from claims which have been made that *p*-aminophenol could be detected and even estimated by this means in urines after administration of sulphanilamide [James, 1939; 1940]. We were of the opinion that the *in vivo* removal of the —SO_2NH_2 from sulphanilamide and, therefore, the formation of *p*-aminophenol was highly improbable. Since, at the time, no claim to the actual isolation of *p*-aminophenol had been made, we decided upon a critical examination of the evidence for its presence. As a preliminary, we investigated the bleaching powder variation of the indophenol reaction, and noticed that sulphanilamide itself gave a positive reaction,[1] although not so strongly as *p*-aminophenol. The reaction of the solution was of importance, the more strongly alkaline the solution the more intense being the colour. A blue colour gradually developed with *p*-aminophenol and phenol in the absence of bleaching powder if the mixture was made alkaline with NaOH, Na_2CO_3 or NH_4OH, although the colours were much intensified by adding bleaching powder solution. A relatively feeble colour could be obtained by treating phenol and ammonia (without *p*-aminophenol) with calcium hypochlorite or bleaching powder solution (this is a well-known test for phenol). Such a test was obviously unsatisfactory for examination of urines containing sulphanilamide, and we have worked out a modification of the test which, while sufficiently sensitive for *p*-aminophenol, is not given by sulphanilamide. This test is performed as follows.

---

[1] With a freshly prepared (acid) solution of calcium hypochlorite a green colour was obtained; in the presence of NaOH or $Ca(OH)_2$ the colour was blue.

To 0·5–1 ml. of neutral or faintly acid test solution 3–4 drops of 5% phenol are added, followed by a few drops of sodium hypobromite solution. With *p*-aminophenol, a blue colour appears, becoming more intense on standing and turning red on acidifying. Sulphanilamide under these conditions gives a pale pink colour and ammonium salts only a very feeble blue colour. The hypobromite solution is prepared by adding bromine to *N* Na_2CO_3 (not NaOH) until the solution is definitely yellow. It should be freshly prepared and discarded when the yellow colour has faded.

In order to detect small amounts of *p*-aminophenol in urine by this or the bleaching powder test, it is necessary to separate the *p*-aminophenol from interfering substances in the urine. This was done by two methods.

(1) *Ether extraction.* After hydrolysis with HCl (25 ml. urine boiled with 20 ml. of 3*N* HCl for 20 min.), the urine was made faintly alkaline with Na_2CO_3 and extracted with ether in a continuous extractor for 2 hr. The indophenol test was performed on the evaporated ether extract.

(2) *Electrodialysis.* 50 ml. urine were hydrolysed by boiling for 20 min. with 1 ml. conc. HCl. The solution was neutralized with NaOH, made up to 100 ml. and placed in the middle compartment of the electrodialysis cell described by MacGregor & Thorpe [1933]. The cathode and anode compartments each held 100 ml. distilled water, and cellophane membranes were used. The cathode was of pure nickel and the anode a carbon plate. A current not exceeding 1 amp. was passed (mains voltage 220). Portions of the cathode liquid were tested at intervals. The test was regarded as negative if no indophenol reaction had been obtained after passing the current for at least 3 hr.

Using one, or more usually both, of these methods, we examined urines from eight patients receiving sulphanilamide and from twelve rabbits heavily dosed (1 g. per kg. body weight) with the drug. In no instance in which sulphanilamide was the only drug administered did we obtain a positive result. Traces of *p*-aminophenol added to urine were easily detected by either method. As a further control we dosed rabbits with acetanilide (0·1 g. per kg. body weight), which is known to be excreted partly as *p*-aminophenyl hydrogen sulphate. Good indophenol tests were obtained from these urines. We had no difficulty in detecting *p*-aminophenol in the urine of one of us who had taken a 'Veganin' tablet containing 0·25 g. phenacetin 4 hr. previously. It is known that *p*-aminophenol is excreted after taking phenacetin [Müller, 1888; Mörner, 1889]. From our experiments we can only conclude that no detectable amount of *p*-aminophenol is formed from sulphanilamide *in vivo*.

After the conclusion of our investigation, James [1940] published a paper in which he claims to have estimated and isolated *p*-aminophenol from the urine of patients receiving sulphanilamide. He states that he estimated *p*-aminophenol by the indophenol reaction. We cannot criticize this since he provides no details. His method of isolation is essentially similar to the ether extraction method which we used, and his identification, though not conclusive, is suggestive.

James's urines were presumably obtained from hospital patients. Since it is common hospital practice to permit the ward sisters to administer antipyretic drugs at their discretion, it occurred to us that James's *p*-aminophenol might be derived from phenacetin taken in such a way. We therefore worked up, according to James's directions, a 24 hr. specimen of the urine of a hospital patient who had received, in addition to sulphanilamide, some 'Veganin' tablets. We obtained an ether extract from the hydrolysed urine at *p*H 9 which gave a strong indophenol reaction.

The Pucher and Day reaction for hydroxylamines

This reaction is based on Bamberger's [1899] observation that hydroxylamine and β-arylhydroxylamines, when treated with benzoyl chloride, produce benzhydroxamic acids [see Cohen, 1931], which form violet ferric salts. Pucher & Day [1926] applied the reaction to the estimation of hydroxylamine itself, but not in urine. As a qualitative test for β-arylhydroxylamines, we believe the reaction to be reliable, the expected result being obtained after reduction by zinc dust of several nitro compounds from which the formation of hydroxylamines could be reasonably inferred. But the test is not specific, and a similar violet colour may be obtained when the test is applied to certain substances which are not hydroxylamines, even if the conditions specified by Pucher and Day for the quantitative estimation are carefully followed.

Several phenols give a violet colour with $FeCl_3$. Under the conditions of the Pucher and Day test, the phenolic hydroxyl should be benzoylated and, therefore, no violet colour would be given with $FeCl_3$. But in certain phenols another substituent group inhibits the reaction between the hydroxyl group and benzoyl chloride, so that a violet colour is produced when $FeCl_3$ is added, even the weak $FeCl_3$ used by Pucher and Day. The simplest example of such a phenol is salicylic acid, which gives a brilliant Pucher and Day reaction. Salicylamide behaves similarly. Another example is 1-amino-3-hydroxybenzene-4-sulphonic acid, which can also give a weak Pucher and Day reaction if the conditions are strictly adhered to. A more intense reaction is obtained if a stronger $FeCl_3$ solution is used (the colour from hydroxylamines is not intensified in this way), whilst with an excess of benzoyl chloride the reaction is negative. Presumably benzoylation of the hydroxyl is inhibited by the adjacent $—SO_3H$, although not to so great an extent as by $—COOH$ or $—CONH_2$. 1-Amino-2-hydroxybenzene-4-sulphonic acid does not give the reaction, the hydroxyl being sufficiently far from the $—SO_3H$ to benzoylate without difficulty. The corresponding aminophenolsulphonamides do not give Pucher and Day's reaction. It is, therefore, essential to prove the absence of phenols which are not benzoylated before a positive Pucher and Day reaction can be taken as evidence for the presence of a hydroxylamine in a mixture of unknown composition such as urine.

We have not used the Pucher and Day test quantitatively except to make the following observation. 2 ml. of a solution of hydroxylamine hydrochloride (100 mg. per 100 ml.) were added to 4 ml. each of water and several different urines (neutral or acid to litmus). The solutions were treated according to Pucher and Day's directions for the quantitative estimation of hydroxylamine, and compared in a comparator backed in each case by the appropriate control (water or diluted urine treated with benzoyl chloride, etc.). The colour from the aqueous hydroxylamine solution was much more intense than those from the urines in most of which the colour was feeble. Further, the colours obtained from different specimens of urine were not the same. We are left with the impression that the Pucher and Day test is quite unsuitable for estimating hydroxylamines in urines unless the hydroxylamine is previously separated from the urine.

For these reasons, we have little confidence in the report by James [1940] that he has estimated in urines, by Pucher and Day's method, the hydroxylamine formed from sulphanilamide. We are, in fact, at a loss to explain how James obtained a positive result at all. We have examined urines from eleven patients and seventeen rabbits dosed with sulphanilamide and have only once obtained a positive reaction.[1] This urine was from a patient who had also taken some acetylsalicylic acid in the form of 'Veganin' tablets. The presence of salicylic acid in this urine was detected by the formation of methyl salicylate. The urine gave a violet colour with $FeCl_3$ alone. James does not record that he tested his urines for salicylates. If the Pucher and Day test is claimed as evidence for the presence of hydroxylamine in human urine, the claim should at least be accompanied by the assurance that the urine gave no colour with $FeCl_3$ alone.

Marshall's diazo reaction applied to sulphanilamide and β-arylhydroxylamines

Sulphanilamide is usually estimated in blood and urine by diazotization with $NaNO_2$ and HCl. After addition of ammonium sulphamate to remove excess HNO_2, the solution is coupled with an alcoholic solution of a naphthylamine derivative, e.g. dimethyl-α-naphthylamine [Marshall *et al.* 1937], and the resulting red colour matched in a colorimeter. Rosenthal & Bauer [1939] called attention to Bamberger's [1898] observation that β-arylhydroxylamines form diazonium salts with nitrous acid, and showed that while the amino group was readily acetylated with acetic anhydride and thus rendered undiazotizable, β-arylhydroxylamines could be diazotized even after treatment with acetic anhydride.[2] They therefore estimated the red diazo colour before and after treatment with acetic anhydride. They claimed that the difference gave the true sulphanilamide value, and that the value obtained after acetylation was due to arylhydroxylamine.

We have tested Rosenthal and Bauer's hydroxylamine reaction by reducing a number of aromatic nitro compounds with zinc dust. The solutions readily diazotized and coupled even after treatment with acetic anhydride and the presence of a hydroxylamine in the solutions was confirmed by the Pucher and Day test. But we also obtained results which led us to doubt the reliability of these tests, and, therefore, their quantitative value in solutions of uncertain composition such as urine.

It is not justifiable to assume that sulphanilamide and *p*-hydroxylaminobenzenesulphonamide are the only compounds present in the urine of a subject who has received sulphanilamide which diazotize and couple. There is the possibility of the presence of a diazotizable aminophenol derivative such as 1-amino-3-hydroxy- or 1-amino-2-hydroxy-benzenesulphonamide. We have, in fact, some evidence that such a compound is present in sulphanilamide urines. If *p*-aminophenol were formed from sulphanilamide—which we do not believe—

---

[1] To eliminate the possibility of the hydroxylamine being destroyed on keeping in urine, we tested some urines from rabbits and one human urine within 15 min. of the sample being voided. No positive results were obtained.

[2] The chemistry of this reaction is far from clear. If an arylhydroxylamine is acetylated it should not diazotize and couple. Bratton *et al.* [1939] have stated, and we have confirmed, that *p*-*N*-acetylhydroxylaminobenzenesulphonamide does not diazotize and couple. (See also Thorpe & Williams [1941].)

it would be partly excreted as *p*-aminophenylsulphuric acid, which can be diazotized and will then couple with dimethyl-α-naphthylamine [James, 1940]; free *p*-aminophenol only does so in concentrated solutions. 1-Amino-2-hydroxybenzene-4-sulphonamide [Thorpe & Williams, 1941], 1-amino-2-hydroxybenzene-4-sulphonic acid [Friedländer, 1911], *o*-aminophenol and 1-hydroxy-2-aminobenzene-4-sulphonic acid (B.D.H.) give, with nitrous acid, a yellow colour which does not become red on adding dimethyl-α-naphthylamine. In all these compounds there is an OH group *ortho* to the NH_2 and diazo-oxide formation is possible [see Hickinbottom, 1936]. If, however, this OH is methylated the red colour is obtained. Methylated solutions of the above phenols were prepared by shaking their aqueous solutions with methyl sulphate and alkali at room temperature: in the case of *o*-aminophenol, *o*-anisidine was used in addition to the methylated aqueous solution of the phenol itself. It appears, therefore, that if any 1-amino-2-hydroxybenzene-4-sulphonamide were formed *in vivo* and excreted conjugated with glucuronic acid and/or sulphuric acid at the OH group, then such conjugates would give the Marshall diazo reaction in addition to sulphanilamide itself.

In certain compounds, a diazo reaction after acetic anhydride treatment can be due to a free amino group, since other groups present may prevent the acetylation of the $—NH_2$. The first example of this which we encountered was 1-nitro-3-aminobenzene-4-sulphonic acid, which cannot be acetylated under the Rosenthal and Bauer conditions, even if a large excess of acetic anhydride be used, so that the 'acetylated' solution of this compound gives a diazo reaction. A similar result might be expected with an *o*-aminophenol conjugated through its —OH with sulphuric or glucuronic acids (see next paragraph), e.g. 1-amino-2-hydroxybenzene-4-sulphonamide, which is a possible detoxication product of sulphanilamide. In a note published some time after Rosenthal and Bauer's paper, Rosenthal [1939, 2] withdrew their claim for specificity and quoted sulphanilamide glucoside (structure not specified) as the compound giving the reaction.

We have examined twenty-four urines (human and rabbit) from subjects treated with sulphanilamide and obtained a positive diazo reaction after acetylation in thirteen cases, eight being very feeble. In none of these urines has the suggestion of an arylhydroxylamine been supported by a positive Pucher and Day test. It can be argued that the diazo test is more sensitive than that of Pucher and Day, and that, therefore, these observations are not incompatible with the presence of an arylhydroxylamine. Against this is the following observation. The only strong reactions were obtained in rabbit urines (presumably owing to the relatively higher dosage). If such urines were boiled with dilute HCl for a few minutes, a diazo reaction after acetylation was no longer given (a solution of *p*-hydroxylaminobenzenesulphonamide still gave the acetylated diazo reaction after boiling with HCl under similar conditions). Such a result could be due to an *o*-aminophenol derivative, e.g. 1-amino-2-hydroxybenzene-4-sulphonamide excreted in conjugation with sulphuric acid. The close proximity of the strongly acidic $—SO_4H$ group to the amino group might prevent acetylation of the latter, and the compound could, therefore, give a Rosenthal and Bauer diazo reaction. After hydrolysis there would be no diazo reaction because 1-amino-2-hydroxybenzene-4-sulphonamide does not couple. In support of this is the fact that the urine before hydrolysis gave no colour with $FeCl_3$, whereas after hydrolysis it gave a red-violet colour.

From the foregoing it is clear that neither the Pucher and Day nor the Rosenthal and Bauer tests can be relied upon for the detection of hydroxylamines in urine. In the absence of a specific reaction, we suggest that the following tests should be applied to the urine.

Test	Expected result if arylhydroxylamine is present
1. Pucher and Day test	Violet colour
2. Marshall's diazo reaction	Red colour
3. Diazo reaction after acetylation (Rosenthal and Bauer)	Red colour
4. Repeat test 2 on the urine after short boiling with HCl	Red colour
5. Add $FeCl_3$ solution	No red or violet colour
6. Repeat test 5 on the urine, after boiling with HCl and neutralizing	No red or violet colour

If *all* these tests give the expected results, there is a probability, but not a certainty, that a β-arylhydroxylamine is present. Tests 2 and 3 would not be given by an arylacetylhydroxylamine. With β-phenylhydroxylamine the result of test 6 is abnormal owing to the formation of *p*-aminophenol. Table 1 gives some typical results with these tests.

Table 1

	Pucher and Day 1	Marshall's diazo 2	Rosenthal and Bauer diazo 3	Marshall's diazo after HCl 4	$FeCl_3$ 5	$FeCl_3$ after HCl 6
β-Phenylhydroxylamine		– *	+	+	–	–
p-Hydroxylaminobenzenesulphonamide	+	–		+	–	–
p-Acetylhydroxylaminobenzenesulphonamide	–	–	–	+	–	+
Salicylic acid	+	–	–	–	+	–
Salicylamide	+	–	–	–	–	+
1-Amino-2-hydroxybenzene-4-sulphonic acid	–	– †	–	–	+	–
1-Amino-2-hydroxybenzene-4-sulphonamide	–	– †		–	–	–
1-Amino-3-hydroxybenzene-4-sulphonic acid	+	–	–	+	–	+
1-Amino-3-hydroxybenzene-4-sulphonamide	–	–	–	–	+	–
1-Nitro-3-aminobenzene-4 sulphonic acid		+	+	+	–	–
1-Nitro-3-aminobenzene-4-sulphonamide	–	+	–	+	–	–
2-Oxybenzoxazole-6-sulphonic acid		–	–	–	–	–
2-Oxybenzoxazole-6-sulphonamide	–	–	–	–	–	–
p-Aminophenol	–	+ ‡	–	+ ‡	–	–
Sulphanilamide	–	–	–	–	–	–

+ Indicates expected colour obtained. – No colour.

* Excess of nitrite may be required to get good reaction.
† Positive after methylation. ‡ Colour feeble and bluish.

SUMMARY

The evidence for the biological formation of arylhydroxylamines is reviewed. It is concluded that there is as yet no reliable evidence for the biological formation of such compounds in the mammal, and, in particular, for the presence of *p*-hydroxylaminobenzenesulphonamide in the urine of humans and rabbits treated with sulphanilamide.

Whilst *p*-aminophenol can be detected in urine after treatment with certain drugs (e.g. acetanilide, phenacetin), there is no reliable evidence that it occurs in urine after treatment with sulphanilamide.

The colour reactions (Rosenthal and Bauer's diazo test, Pucher and Day's test, and the indophenol test) which have been used for identification of these substances have been examined and shown unreliable for this purpose.

We wish to thank Dr A. R. McWhinney, Dr G. E. Murphy, Miss J. Pitt, Mr Garfield Thomas and Dr Margaret Thorpe for obtaining for us specimens of urine from sulphanilamide-treated patients.

REFERENCES

Bamberger (1898). *Ber. dtsch. chem. Ges.* **31**, 574.
—— (1899). *Ber. dtsch. chem. Ges.* **32**, 1805.
Binet (1889). Cited by Hinsberg & Treupel (1894). *Arch. exp. Path. Pharmak.* **33**, 217.
Böhm (1939). *Hoppe-Seyl. Z.* **261**, 35.
Bratton, White & Marshall (1939). *Proc. Soc. exp. Biol., N.Y.*, **42**, 847.
Brownlee (1939). *Biochem. J.* **33**, 697.
Cohen (1931). Organic Chemistry, Vol. II, 305. London.
Ellinger (1920). *Hoppe-Seyl. Z.* **111**, 86.
Fox (1940). *Amer. J. med. Sci.* **199**, 487.
Friedländer (1911). '*Fortschr. Teerfarb.*' **9**, 148 (D.R.P. 197,496).
Gray, Buttle & Stephenson (1937). *Biochem. J.* **31**, 726.
Herrick & Irons (1906). *J. Amer. med. Ass.* **46**, 351.
Hickinbottom (1936). Reactions of Organic Compounds, p. 285. London.
Horn (1936, 1). *Hoppe-Seyl. Z.* **238**, 84.
—— (1936, 2). *Hoppe-Seyl. Z.* **242**, 23.
—— (1937). *Hoppe-Seyl. Z.* **249**, 82.
Jaffé & Hilbert (1888). *Hoppe-Seyl. Z.* **12**, 295.
James (1939). *Biochem. J.* **33**, 1688.
—— (1940). *Biochem. J.* **34**, 640.
Kleine (1896–7). *Hoppe-Seyl. Z.* **22**, 327.
Lipschitz (1920). *Hoppe-Seyl. Z.* **109**, 189.
MacGregor & Thorpe (1933). *Biochem. J.* **27**, 1394.
Marshall, Emerson & Cutting (1937). *J. Amer. med. Ass.* **108**, 953.
Mayer (1937). *Bull. Acad. Méd., Paris*, **117**, 727.
Mörner (1889). *Jber. Fortschr. Tierchem.* **19**, 80.
Müller (1888). *Ther. Monatsh.* 66.
Pucher & Day (1926). *J. Amer. chem. Soc.* **48**, 672.
Rimington & Hemmings (1939). *Biochem. J.* **33**, 960.
Rosenthal (1939, 1). *J. Amer. med. Ass.* **113**, 1710.
—— (1939, 2). *J. Amer. med. Ass.* **113**, 2169.
—— & Bauer (1939). *Publ. Hlth Rep., Wash.*, **54**, 1880.
Scudi (1940). *Science*, **91**, 486.
Shelswell & Williams (1940). *Biochem. J.* **34**, 528.
Sieberg (1916). *Hoppe-Seyl. Z.* **97**, 53.
Thorpe & Williams (1941). *Biochem. J.* **35**, 61.

9. STUDIES IN DETOXICATION

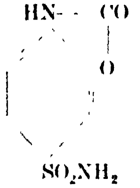
9. THE SYNTHESIS OF SOME POSSIBLE BIOLOGICAL OXIDATION PRODUCTS OF SULPHANILAMIDE

By W. V. THORPE and R. TECWYN WILLIAMS

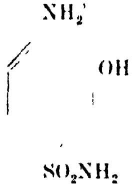
From the Physiology Department, Medical School, Hospitals Centre, Birmingham

(*Received 19 December 1940*)

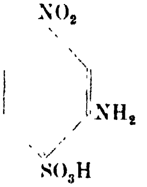
In the previous paper [Thorpe & Williams, 1941] we expressed the opinion that 1-amino-2-hydroxy-(II) and 1-amino-3-hydroxy-benzene-4-sulphonamide (V) and 2-oxybenzoxazole-6-sulphonamide (I) might be biological oxidation products of sulphanilamide. Here we describe the synthesis of these compounds. No difficulty was experienced in the preparation of the *o*-aminophenol and benzoxazole derivatives which were obtained at different stages in the same synthesis, but the synthesis of the *m*-aminophenol derivative presented unexpected difficulties:



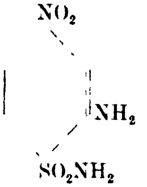
I



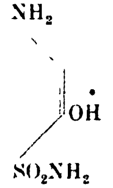
II



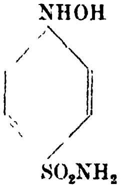
III



IV



V



VI

We first attempted a synthesis of 1-amino-3-hydroxybenzene-4-sulphonamide (V), starting with the easily prepared *m*-nitraniline-sulphonic acid (III). It was hoped to obtain large amounts of 1-nitro-3-aminobenzene-4-sulphonamide (IV), and thence, by diazotization and reduction, the required *m*-aminophenol-sulphonamide. We found, however, after many trials using different chlorinating agents, that the nitranilinesulphonic acid formed little or none of the corresponding sulphonyl chloride. Small amounts of (IV) were eventually obtained in one experiment using chlorosulphonic acid, but this result could not be repeated. This proposed method of synthesis was therefore abandoned. One interesting point which emerged at this stage was that ammonium 1-nitro-3-aminobenzene-4-sulphonate was isolated in a dimorphous state, a pale orange and a red form. The orange form could be easily converted into the red form by gentle warming below 100°. The red form, once formed, was apparently stable.

Success was eventually attained starting with *m*-aminophenolsulphonic acid, which is easily prepared by heating *m*-aminophenol with conc. H_2SO_4. The synthesis worked smoothly to the sulphonyl chloride stage, whilst the final stages were completed with some difficulty.

Although many workers (see previous paper for literature) state that they have used *p*-hydroxylaminobenzenesulphonamide (VI) in biological work, no mention is made in their papers of its method of preparation or chemical properties. This substance has been prepared by Bratton *et al.* [1939] and we have repeated their process and agree with their description of its chemical properties.

We have also prepared its *N*-acetyl derivative[1] which has M.P. 227° and gives no diazo reaction in agreement with Bratton *et al.* [1939]. We mention this since James [1940] claims to have isolated this acetyl compound from human urine after sulphanilamide medication, and states that the substance he isolated did not melt below 300°. We failed to prepare *p*-hydroxylaminobenzenesulphonamide using Bamberger's [1894] method with zinc dust at low temperatures, and Haworth & Lapworth's [1921] sodium hydrosulphide method for the preparation of arylhydroxylamines.

The phenolic sulphonamides (II) and (V) have not yet been tested for therapeutic activity. Of phenolic sulphonamides which have been tested for therapeutic activity, 1-hydroxy-2-aminobenzene-4-sulphonamide [Tréfouel *et al.* 1937; McLeod, 1938] and *p*-hydroxybenzenesulphonamide [Buttle *et al.* 1936; McLeod, 1938] were inactive, and 1-hydroxy-3-nitrobenzene-4-sulphonamide [McLeod, 1938] was active. Compounds related to our 1-nitro-3-aminobenzene-4-sulphonamide have also been tested and found inactive, namely 1-nitro-4-aminobenzene-3-sulphonic acid [Buttle *et al.* 1936] and 1-nitro-6-aminobenzene-3-sulphonamide [Tréfouel *et al.* 1937].

EXPERIMENTAL

2-Oxybenzoxazole-6-sulphonyl chloride. Benzoxazolone (2-oxybenzoxazole) was prepared from salicylamide and NaOCl according to Graebe & Rostovzeff [1902]. The only modification introduced was that the benzoxazolone was precipitated from the alkaline reaction mixture by neutralizing carefully, with cooling, with conc. HCl instead of by passing CO_2 into the solution. The neutralization with CO_2 may take several hours (on a 20 g. scale) whilst with HCl it can be done in under half an hour. Sodium benzoxazolonesulphonate was prepared according to the patent directions [Friedländer, 1911]. The dry salt (10 g.) was stirred with 12 g. PCl_5. The mass warmed up and became pasty, and after standing half an hour with occasional stirring, it was treated with ice water in the usual manner. The precipitated *chloride* was filtered, washed with water and dried (yield 6 g.). It crystallized from toluene in colourless rhombic plates, M.P. 186–187°.[2] (Found: C, 36·2; H, 1·8; N, 6·1 %. $C_7H_4O_4NClS$ requires C, 36·0; H, 1·7; N, 6·0 %).

2-Oxybenzoxazole-6-sulphonamide (I) was prepared by adding the powdered chloride to strong ammonia. The deposit formed overnight was filtered and dissolved in warm dilute NaOH. On cooling, the *sodium salt* of the sulphonamide separated as needles, which were recrystallized from water. (Found: N, 10·2; Na, 8·4; H_2O, 13·4 %. $C_7H_5O_4N_2SNa, 2H_2O$ requires N, 10·3; Na, 8·45; H_2O, 13·2 %.) The *sulphonamide* itself immediately separates as needles if an aqueous solution of the sodium salt is acidified. On recrystallization from water, the sulphonamide formed needles, M.P. 263°. (Found: C, 39·4; H, 2·8; N, 13·3 %. $C_7H_6O_4N_2S$ requires C, 39·25; H, 2·8; N, 13·1 %.)

1-Amino-2-hydroxybenzene-4-sulphonamide (II). 2·8 g. of the foregoing sodium salt were boiled for 20 min. with 25 ml. of 30 % NaOH. Crystals of

---

[1] We experienced some difficulty in this acetylation. Using acetic anhydride as recommended by Bratton *et al.* we persistently obtained a product melting about 160° from which Bratton's acetyl derivative was only obtained in small yield by fractional crystallization. We did not examine the other product in detail except to note that it gave a diazo reaction, and prove that it was not unchanged *p*-hydroxylaminobenzenesulphonamide. It may be the substance responsible for Rosenthal and Bauer's diazo reaction (see Thorpe & Williams [1941]).

[2] All melting-points are uncorrected.

Na_2CO_3 separated in the solution, and were liable to cause violent bumping. The solution was cooled, carefully neutralized with conc. HCl and then made alkaline with solid Na_2CO_3. The solution was now extracted for 8 hr. with ether in a continuous extractor and on evaporation of the ether the *sulphonamide* was obtained as a crystalline residue (2 g.). The compound was purified by dissolving in hot absolute alcohol and adding sufficient carbon tetrachloride to produce a faint cloudiness: on cooling the sulphonamide separated as clusters of long needles, M.P. 164°. It was soluble in water and alcohol, sparingly soluble in ether and insoluble in CCl_4 It reduced ammoniacal silver nitrate on warming and gave a purple colour with $FeCl_3$ turning a deep red in about 30 sec. It slowly caused the formation of methaemoglobin in a 1 in 50 solution of blood at 37°. (Found: C, 38·6; H, 4·3; N, 15·6%. $C_6H_8O_3N_2S$ required C, 38·3; H, 4·3; N, 14·9%.) The *hydrochloride* was prepared by adding the requisite amount of conc. HCl to an alcoholic solution of the sulphonamide. On stirring the solution the hydrochloride separated; more alcohol was now added and the solution boiled until the hydrochloride dissolved. Carbon tetrachloride was then poured into the solution until the hydrochloride just began to crystallize and the crystallization was completed at 0°. It formed fine matted needles, which were very soluble in water and which decomposed without melting above 300°. It was purified by crystallization from absolute alcohol and CCl_4. (Found: C, 32·3; H, 3·9: N, 12·6%. $C_6H_8O_3N_2S.HCl$ requires C, 32·1; H, 4·0; N, 12·5%.)

Treatment of an aqueous solution of the sulphonamide with benzoyl chloride and NaOH gave *1-benzamino-2-benzoxybenzene-4-sulphonamide*, which formed needles from aqueous alcohol, M.P. 191°. (Found: C, 60·0; H, 3·9; N, 7·4%. $C_{20}H_{16}O_5N_2S$ requires C, 60·6; H, 4·1; N, 7·1%.)

1-Amino-3-hydroxybenzene-4-sulphonic acid was prepared by sulphonation of *m*-aminophenol [cf. Friedlander, 1896]. 15 g. of *m*-aminophenol were heated on the water bath with 25 ml. conc. H_2SO_4 and a few drops of fuming H_2SO_4 for an hour. The solid product was broken up in about 150 ml. of water and 10% NaOH added with stirring until a clear solution was obtained. The solution was now filtered, acidified with HCl and set aside to crystallize at 0°. The acid (tablets) was filtered, washed with water and dried over $CaCl_2$: yield 24 g.

Pyridine 1-acetamino-3-acetoxybenzene-4-sulphonate. The above acid (10 g.) was shaken with 25 ml. pyridine and then 25 ml. acetic anhydride added. Addition of the anhydride caused the evolution of heat: the solution was therefore cooled under the tap. The acid dissolved in about an hour, the solution being occasionally shaken to facilitate solution. The mixture solidified on keeping at room temperature overnight. The solid was filtered under suction, pressed and washed with small amounts of methylated spirit. It was recrystallized from spirit, ether being added to facilitate the crystallization. On evaporating the mother liquor from the acetylation mixture *in vacuo*, a small amount of the salt was recovered: yield 15 g. M.P. 170–172°. The substance gave no colour with $FeCl_3$ and no diazo reaction. (Found: N, 7·9%. $C_{15}H_{16}O_6N_2S$ requires N, 7·95%.)

1-Acetamino-3-acetoxybenzene-4-sulphonyl chloride. The foregoing pyridine salt (5 g.) was stirred with 5 g. PCl_5. The mixture soon became pasty and warm. After 15–20 min., the mixture was heated for a few minutes on the water bath. The product was then thrown into ice-water and, on stirring, the *chloride* separated as a white solid (yield almost quantitative). It crystallized from toluene as long rods, M.P. 169°. (Found: C, 41·4; H, 3·4; N, 4·85%. $C_{10}H_{10}O_5NClS$ requires C, 41·2; H, 3·45; N, 4·8%.)

1-Acetamino-3-hydroxybenzene-4-sulphonamide. The above chloride was added to 5 times its weight of 40% ammonia (40 ml. NH_4OH sp.gr. 0·880 made up to *100* ml. *with water*). The chloride gradually dissolved, the solution becoming warm. After an hour the opalescent solution was filtered and evaporated to a thin paste on the water bath. After cooling, the paste was stirred with about 5 vol. water and the sparingly soluble crystalline material collected and washed with water. The *1-acetamino-3-hydroxybenzene-4-sulphonamide* was recrystallized from 50 % aqueous alcohol as colourless needles, M.P. 280° decomp. (The melting-point was observed after raising the temperature fairly rapidly up to about 270°; if heated very slowly decomposition sets in about 250°, observed melting-point may be as low as 265° and it is difficult to get consistent results.) Yield of crude product 30 % weight of chloride. The pure substance gave no diazo reaction. A violet colour with $FeCl_3$ was obtained with a solution of the amide in 50 % aqueous alcohol. (Found: C, 41·8 %; H, 4·5; N, 12·2 %. $C_8H_{10}O_4N_2S$ requires C, 41·75; H, 4·4; N, 12·2 %.)

The mother liquor from the separation of this amide gave on further evaporation crystalline material (yield up to 10 % weight of chloride) rather more soluble than the main product. It crystallized from 30 % aqueous alcohol in apparently homogeneous needles, M.P. 235°. It gave no diazo reaction, but readily gave a violet colour with $FeCl_3$ with an aqueous solution. On hydrolysis with 2*N* NaOH, it yielded 1-amino-3-hydroxybenzene-4-sulphonamide. C, H and N values (C, 42·6; H, 4·5; N, 11·1 %) gave no clue to the nature of this substance (mixture?) and we did not investigate it further.

The filtrate from the separation of the unidentified substance appeared to contain small amounts of this unidentified substance or the above amide (or both) and also some acetylated 1-amino-3-hydroxybenzene-4-sulphonic acid, since after hydrolysis with 2*N* NaOH some 1-amino-3-hydroxybenzene-4-sulphonamide could be obtained by extraction with ether, and 1-amino-3-hydroxybenzene-4-sulphonic acid (yield up to 10 % weight chloride) separated in tablets on acidifying with HCl.

1-Amino-3-hydroxybenzene-4-sulphonamide (*V*). 1-Acetamino-3-hydroxybenzene-4-sulphonamide (1 g.) was dissolved in 10 ml. 2*N* NaOH and heated in a boiling water bath for 3½ hr. Water lost by evaporation was replaced at intervals. (Hydrolysis with 2*N* HCl was equally effective.) The mixture was then made neutral or just acid by addition of conc. HCl and finally rendered just alkaline by addition of solid Na_2CO_3. The solution was extracted with ether in a continuous extractor for at least 8 hr. *1-Amino-3-hydroxybenzene-4-sulphonamide* separated from the extract in clusters of long colourless plates (yield 0·5 g.). A further crop (0·1 g.) was obtained by evaporation of the ether, but the product obtained in this way was liable to turn brown on keeping unless it was immediately purified. The amide was recrystallized by taking up in a small amount of absolute alcohol followed by addition of CCl_4 until cloudy. The amide gradually crystallized in long plates which had M.P. 152° and gave a strong diazo reaction and a weak red-violet colour with $FeCl_3$. It was readily soluble in water and alcohol, sparingly soluble in ether, insoluble in CCl_4. (Found: C, 38·7; H, 4·4; N, 14·6 %. $C_6H_8O_3N_2S$ requires C, 38·3; H, 4·3; N, 14·9 %.) On adding conc. HCl to a concentrated aqueous solution, the *hydrochloride* separated in elongated rhombic plates which could be recrystallized from a small volume of 80 % aqueous alcohol. By shaking an aqueous solution of the amide with acetic anhydride, the monoacetyl derivative M.P. 280° already described separated from the solution. The amide did not cause formation of methaemoglobin when incubated with a 1 in 50 blood solution.

1-Nitro-3-aminobenzene-4-sulphonamide (*IV*). *m*-Nitraniline was sulphonated according to Friedländer [1923] and 1-nitro-3-aminobenzene-4-sulphonic acid isolated as the sparingly soluble sodium salt. Treatment of the dry Na salt or of the free acid itself with PCl_5, thionyl chloride or chlorosulphonic acid failed to give any indication of the formation of the sulphonyl chloride except in one case. An account of this isolated case is given, but we must emphasize that this experiment could not be repeated. 20 g. of the dry *m*-nitranilinesulphonic acid were heated on the water bath for 3·5 hr. with 20 ml. of chlorosulphonic acid. The resulting thick liquid was poured into ice-water with stirring. A yellow precipitate separated which was filtered and washed twice with water. After pressing as dry as possible, it was stirred into 200 ml. ammonia (sp. gr. 0·880), in which it immediately dissolved. After 24 hr. at room temperature a deposit (6 g.) of clear light brown prisms of large size separated, having M.P. 213–214°. On evaporating the mother liquor *ammonium 1-nitro-3-aminobenzene-4-sulphonate* was obtained. This salt crystallized at room temperature as large orange prisms, but if the temperature of crystallization was above room temperature, then a mixture of bright red and orange crystals was obtained. The orange form could be easily converted into the red form on heating in a test tube at about 80°. The red form once formed appeared to be stable. (Found, orange form: C, 30·8; H, 3·8; N, 17·6 %: red form, C, 30·5; H, 3·8; N, 17·8 %. $C_6H_9O_5N_3S$ requires C, 30·6; H, 3·9; N, 17·9 %.) The light brown crystals of M.P. 213–214° appeared to be mainly *1-nitro-3-aminobenzene-4-sulphonamide*, contaminated with some of the above ammonium salt. The crystals were recrystallized from water and formed yellow prismatic needles, M.P. 215°. (Found: C, 33·3; H, 3·2 %. $C_6H_7O_4N_3S$ requires C, 33·2; H, 3·25 %.)

SUMMARY

The synthesis of 1-amino-2-hydroxybenzene-4-sulphonamide, 2-oxybenzoxazole-6-sulphonamide, 1-amino-3-hydroxybenzene-4-sulphonamide and several related compounds is described. Their preparation was necessitated because they may be biological oxidation products of sulphanilamide.

1-Amino-2-hydroxybenzene-4-sulphonamide is a methaemoglobin-former *in vitro*, but 1-amino-3-hydroxybenzene-4-sulphonamide is not.

We wish to express our gratitude to Prof. W. N. Haworth and Dr M. Stacey, who arranged for the C, H and some of the N estimations to be done by their micro-analyst.

REFERENCES

Bamberger (1894). *Ber. dtsch. chem. Ges.* **27**, 1548.
Bratton, White & Marshall (1939). *Proc. Soc. exp. Biol., N.Y.*, **42**, 847.
Buttle, Gray & Stephenson (1936). *Lancet*, **1**, 1289.
Friedländer (1896). *Fortschr. Teerfarb.* **3**, 59 (D.R.P. 71,229).
—— (1911). *Fortschr. Teerfarb.* **9**, 148 (D.R.P. 197,496).
—— (1923). *Fortschr. Teerfarb.* **13**, 242 (D.R.P. 294,547).
Graebe & Rostovzeff (1902). *Ber. dtsch. chem. Ges.* **35**, 2751.
Haworth & Lapworth (1921). *J. chem. Soc.* **119**, 768.
James (1940). *Biochem. J.* **34**, 640.
McLeod (1938). *Biochem. J.* **32**, 1770.
Thorpe & Williams (1941). *Biochem. J.* **35**, 52.
Tréfouel, Tréfouel, Nitti & Bovet (1937). *Ann. Inst. Pasteur*, **58**, 39.

10. PRODUCTION OF AMINES BY BACTERIA

4. THE DECARBOXYLATION OF AMINO-ACIDS BY ORGANISMS OF THE GROUPS *CLOSTRIDIUM* AND *PROTEUS*

By ERNEST FREDERICK GALE[1]
From the Biochemical Laboratory, Cambridge

WITH AN ADDENDUM BY G. L. BROWN, F. C. MACINTOSH
AND P. BRUCE WHITE

(*Received 29 November 1940*)

THE previous papers of this series have dealt with the production of amines by strains of *Bact. coli* [Gale, 1940, 1] and *Strep. faecalis* [Gale, 1940, 2, 3] and with the properties of the amino-acid decarboxylases involved. The present communication continues the study for members of the strictly anaerobic group of *Clostridia*. Results obtained with members of the *Proteus* group are also included, but it will appear that these organisms are not important amine-producers.

Kendall & Schmidt [1926] found that cultures of *Cl. welchii*, when grown in a medium containing glucose, produce a substance in the medium which has the physiological reactions of histamine. Later Kendall & Gebauer [1930] isolated a substance from a bulk culture which had the chemical properties of histamine. Eggerth [1939], in his investigations on the production of histamine by bacteria in culture, obtained positive results with four strains of *Cl. welchii* and most accounts of the toxins of this organism now mention the formation of a 'non-specific histamine-like toxin'. Apart from these instances there appears to be no mention of amine-production by *Clostridia* in the literature. The amino-acid metabolism of certain of the strict anaerobes has been studied: *Cl. sporogenes* by Stickland [1934: 1935], Woods [1936], Kocholaty & Hoogerheide [1938]: *Cl. tetanomorphum* by Woods & Clifton [1937: 1938], and *Cl. botulinum* by Clifton [1940].

Methods

The general technique of preparation of washed suspensions, determination of their dry weight and the manometric investigation of their power to decarboxylate amino-acids has been described fully in the previous papers [Gale, 1940, 1, 2]. In the present survey, the organisms were grown overnight in 800 ml. 2 % glucose broth (tryptic digest of casein), the total organisms obtained made into washed suspension and tested against the 16 amino-acids previously described; for the first experiment in each case, washed suspensions containing 20–30 mg. bacteria/ml. were used so that small activities could be detected, and the suspensions later diluted to extents indicated by the preliminary test. The optimum *p*H was determined in every case and the experiment carried out, unless otherwise stated, at a thermostat temperature of 30°. Decarboxylase activity is measured in terms of Q_{CO_2} as before and values < 1 are regarded as not significant.

The *Clostridia* were kept in most cases in tubes of broth and heart muscle. In the case of *Cl. welchii* a 'cellar' of alkaline egg medium was also maintained, the organism being subcultured into meat-broth before use. It was found, however, that the organism lost activity after 5–6 serial cultivations in broth and so cultures were taken afresh from the egg medium once a week. For experimental purposes the medium used consisted of tryptic digest of casein with a little heart muscle and 2 % additional glucose. This was inoculated with the contents of a cultured tube and incubated at 37° overnight in a Fildes and MacIntosh anaerobic jar. In all cases the organisms were examined microscopically before use to determine the extent to which sporing had occurred; in most cases cultures of 12–15 hr. consisted almost entirely of vegetative cells, exceptions being *Cl. septique* and *Cl. tetanomorphum* where considerable numbers of spores were sometimes encountered. Most of the *Clostridia* grow very thickly in the medium described, growth ceasing after 8–12 hr. when the culture has reached 0·75–0·95 mg. dry weight of bacteria/ml. medium in many cases. *Cl. septique* again forms an exception in that the onset of sporing seems to limit the growth to approx. 0·3 mg./ml. On harvesting, the culture was poured through glass wool to remove fragments of meat and then centrifuged and washed as usual. A few strains of *Cl. welchii* proved difficult to centrifuge cleanly owing to 'roping' of the culture, but this difficulty was only marked with the strains S.R. 9 and Adams, in which cases it was necessary to rely on serial concentration of the suspension rather than on clean separation of the organisms. All washed suspensions were used on the day on which they were prepared, but it was not found necessary to take any special measures to keep them active with regard to the decarboxylases over a period of 4–7 hr.

---

[1] Senior Student of the Royal Commission for the Exhibition of 1851.

Organisms and strains used

Cl. welchii S.R. 9. Classical strain used for toxin production. Isolated from war wound. Toxic type A.

Cl. welchii T.W. 14. Isolated from case of puerperal fever. Type A.

Cl. welchii Chelsea. Probably atoxic strain isolated from the urine of a pregnant woman.

Cl. welchii Fisher. Isolated from war wound. Type A.

Cl. welchii Adams. Very toxic strain from war wound. Type A.

Cl. welchii B.W. 21. Isolated from war wound by Prof. MacIntosh. Type A.

Cl. welchii S. 107. Isolated from sheep faeces. Type A.

Cl. paludis Wye 4. *Welchii*-type organism causing sheep disease. Type B.

Lamb dysentery U. 15. *Welchii*-type organism. Type C.

Cl. septique Pasteur III. Original strain isolated by Pasteur.

All the above organisms and also *Cl. septique* Stokes and Jerral were obtained from Dr M. Robertson to whom my thanks are due for advice on the maintenance of these cultures. I am indebted to Dr Carruthers of the Emergency Public Health Laboratory Service for strains of *Cl. septique*, the non-toxigenic strain of *Cl. tetanum* Fildes and for the various *Proteus* organisms mentioned. All other organisms mentioned below were obtained from the National Collection of Type Cultures and are referred to by their collection number.

Decarboxylation of amino-acids by organisms of the groups Clostridium *and* Proteus

The general conditions under which certain amines are produced by bacteria have been elucidated in the previous papers of this series. In the present work it has been the custom first to undertake a survey of the decarboxylating activities of several strains of each organism when grown in glucose broth and then to choose the most active strain for detailed investigation. As a result of this survey with the two groups of organisms it has been found that only four amino-acids are attacked under the usual experimental conditions and Table 1 shows the activities of the various positive organisms. In each case the Q_{CO_2} value is given for the activity at the optimal *p*H (see below) and 30° and represents the initial steady velocity of simple decarboxylation of the amino-acid in question. The decarboxylation is quantitative in every case since the theoretical volume of CO_2 is obtained within experimental error on completion; the decarboxylation is not accompanied by any other reaction so that the product is the amine corresponding to the amino-acid substrate and this is confirmed by isolation and identification of the product as shown later. For this preliminary work the organisms were grown overnight in the 2 % glucose broth medium described and the *p*H of the medium measured potentiometrically before harvesting as a measure of the saccharolytic activity of the organisms concerned.

Table 1. *Decarboxylation of amino-acids by various* Clostridium *and* Proteus *organisms grown in 2 % glucose broth at 37°. Activities expressed as* Q_{CO_2} *obtained with washed suspensions at 30° and the optimum* pH *in each case*

Organism	Strain	Final *p*H in medium	Q_{CO_2} Histidine	 Ornithine	 Tyrosine	 Glutamic acid
Cl. welchii	S.R. 9	4·6	34	—	—	70
Cl. welchii	T.W. 14	4·5	3	—	—	237
Cl. welchii	Chelsea	4·5	30	—	—	368
Cl. welchii	Fisher	4·6	29			100
Cl. welchii	Adams	4·6	27			116
Cl. welchii	B.W. 21	4·1	60	—	—	132
Cl. welchii	S. 107	4·2	23			50
Cl. paludis	Wye 4	4·6	26	—	—	36
Lamb dysentery	U. 15	4·6	6	—	—	40
Cl. septique	Carr.	5·3	—	180		
Cl. septique	Pasteur	5·3	—	270	—	—
Cl. septique	Stokes	6·0	—	176	—	—
Cl. septique	Jerral	5·75	—	70	—	—
Cl. aerofoetidum	505	5·3	—	—	50	325
Cl. fallax	Morcom	5·1	14	—		—
Cl. fallax	2907	5·05	—	—	—	—
Cl. bifermentans	2914	5·2	8	—		80
Cl. bifermentans	536	5·4	—	—		30
Pr. vulgaris	Fildes	5·25	—	—	—	324
Pr. vulgaris	Crook	5·63	—	9		112
Pr. vulgaris	Carr.	4·85	—	2	—	46
Pr. morganii	Carr.	5·25	—	12	—	134
Pr. morganii	Carr.	5·8	—	16	—	—

Proteus organisms HX 2, HXL 11, HX 19, etc., also attacked glutamic acid, while Kingsbury strains apparently possessed no decarboxylating enzymes. The following members of the *Clostridium* group showed no decarboxylating activities under the usual experimental conditions:

Cl. sporogenes Bellette 533 and 2911.
Cl. histolyticum Weinberg and 6282 (American).
Cl. chauvoei 287 and 2721.
Cl. oedematiens (*novyi*) 2908 and Cossard 277.
Cl. tetanum Fildes.
Cl. butyricum Weizmann 619.
Cl. tertium 336.
Cl. multifermentans-tenalbus 2032.
Cl. tetanomorphum 500 and 2909.
Cl. sphenoides 507.

The organisms chosen for detailed investigation were *Cl. welchii* B.W. 21, *Cl. septique* Pasteur III, *Cl. aerofoetidum* 505, *Cl. fallax* Morcom, *Cl. bifermentans* 2914 and *Proteus morganii*; the results set out below were obtained chiefly with these selected strains.

Properties of the amino-acid decarboxylases

l(−)-*Histidine decarboxylase.* Kendall & Schmidt [1926] reported that 66 strains out of 72 of *Cl. welchii* tested produced a histamine-like substance when grown in the presence of glucose. In Table 1 it can be seen that all the strains used in washed suspension by the author produce histamine under the appropriate conditions. One strain, not included in Table 1, attacked glutamic acid rapidly but failed to decarboxylate histidine, but since this strain has also proved abnormally erratic in its power to produce specific toxin, it has been omitted. Kendall & Schmidt state that certain of their strains lost the power to produce histamine on continued subcultivation and it has been mentioned above that the author found it necessary to return to the alkaline egg medium once a week in order to maintain activity. Histidine is also decarboxylated, but at a slower rate, by some strains of *Cl. fallax* and *Cl. bifermentans.* Gale [1940, 1] showed that strains of *Bact. coli* in washed suspension will decarboxylate histidine quantitatively to histamine at an optimal *p*H of 4·0. When testing the power of *Cl. welchii* to carry out this reaction at various *p*H values, it became obvious that 4·0 is not the optimum *p*H in this case. Fig. 1 shows the variation of histidine decarboxylase activity with reaction *p*H for strains of *Cl. welchii, Cl. fallax* and *Cl. bifermentans.* The optimal activity is obtained between *p*H 2·5 and 3·0 in all cases: *Cl. welchii* B.W. 21 appears to have a well-defined optimum at 2·5 while that of the S. 107 strain lies nearer 3·0. The Q_{CO_2} figures for *p*H values <2 can only be regarded as approximate as the HCl-phthalate mixtures used are not very effective buffers over this part of the range and calculations were accordingly made over a short period of activity, the *p*H being measured immediately before and after this period. The *p*H-activity curve is markedly different from that obtained for the decarboxylation of histidine by *Bact. coli* [Gale, 1940, 1] which has an optimum at *p*H 4·0 in all cases tested. The curve obtained with the coliform organisms falls steeply on the acid side of *p*H 4·0, the enzyme being almost inactive at *p*H 3·5 and it has been suggested that this is due to denaturation of the enzyme protein while in actual practice it is often found that the organisms coagulate below *p*H 4·0. This is not the case with *Cl. welchii* where coagulation of the organisms does not take place even at *p*H 1 and the *p*H-activity curve for the histidine decarboxylase is approximately symmetrical. Thus the difference in the apparent properties of the two enzymes may be due to the greater resistance to denaturation of the proteins of *Cl. welchii* over those of the coliform organisms, but the question is not likely to be solved until both enzymes are obtained in a cell-free state.

l(+)-*Ornithine decarboxylase.* Ornithine is decarboxylated rapidly by all four of the *Cl. septique* strains tested and much more slowly by *Proteus morganii* and some strains of *Pr. vulgaris.* Fig. 2 shows the variation of ornithine decarboxylase activity of some of these three organisms with reaction *p*H. The *Cl. septique* strains show a well-marked optimum at *p*H 5·5, the most alkaline value obtained for an amino-acid decarboxylase up to the present. The ornithine decarboxylase of *Bact. coli* has an optimum at *p*H 5·0 [Gale, 1940, 1].

l(−)-*Tyrosine decarboxylase.* Tyrosine is decarboxylated comparatively slowly by *Cl. aerofoetidum* at an optimum *p*H of 5·0 as shown by Fig. 3. Unfortunately it has been possible to obtain only one strain of this organism. The pH optimum for this one strain is the same as that found for *Strep. faecalis* [Gale, 1940, 2], but whereas that organism attacked only tyrosine, *Cl. aerofoetidum* also decarboxylates glutamic acid.

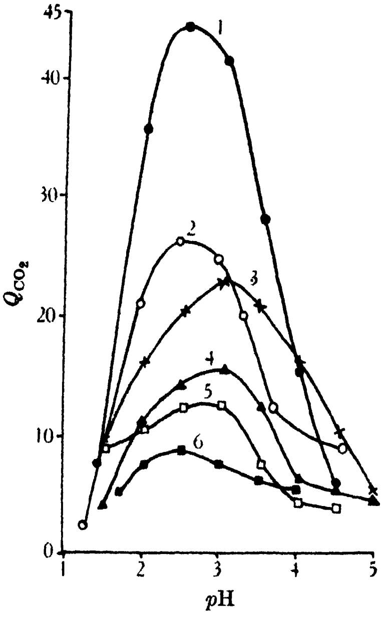


Fig. 1.

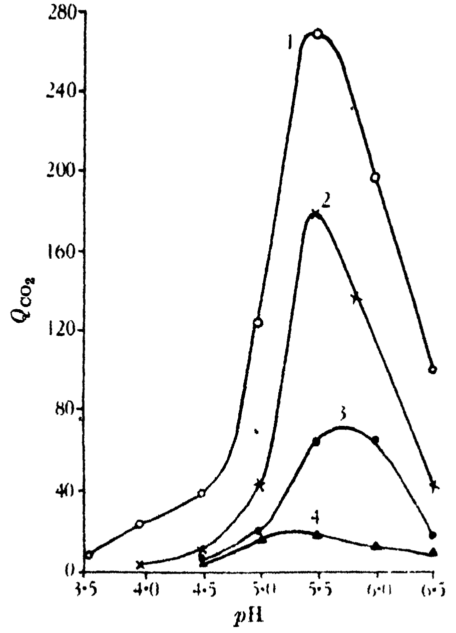


Fig. 2.

Fig. 1. Variation of histidine decarboxylase activity (Q_{CO_2}) with pH. 1, *Cl. welchii* B.W. 21. 2, *Cl. welchii* Chelsea. 3, *Cl. welchii* S. 107. 4, *Cl. welchii* S.R. 9. 5, *Cl. fallax* Morcom. 6, *Cl. bifermentans.*

Fig. 2. Variation of ornithine decarboxylase activity (Q_{CO_2}) with pH. 1, *Cl. septique* Pasteur III. 2, *Cl. septique* Carruthers. 3, *Cl. septique* Jerral. 4, *Pr. morganii.*

l(+)-*Glutamic acid decarboxylase.* Glutamic acid is attacked by all the strains of *Cl. welchii*, *Cl. bifermentans* and *Cl. aerofoetidum* tested and also by the majority of *Proteus* and coliform organisms. Fig. 4 gives the variation of the decarboxylase activity with pH in a selection of these cases; the pH optimum lies between 3·5 and 4·5, usually at 4·0. In all cases the pH-activity curve shows a rapid drop on the acid side of the optimum. In these studies of the decarboxylating powers of various bacteria, the glutamic acid enzyme has been the most frequently encountered and, in general, the most active of the amino-acid decarboxylases investigated. A quantitative study of the CO_2 liberated shows that only one of the —COOH groups of the glutamic acid molecule is removed and, as is shown below, the product has always proved to be γ-aminobutyric acid. It may be that γ-aminobutyric acid plays some important part in the cell metabolism.

Thermolability. In the original investigations with *Bact. coli* it was found that the activity of the washed suspensions fell off rapidly and that this loss was increased with rise of temperature. This thermolability affects all these investigations but it varies greatly from organism to organism and is not constant for any one enzyme; for instance, the glutamic acid decarboxylase of *Bact. coli* is found to be very sensitive to temperature, but the similar enzyme of *P. morganii* is far less sensitive and, when working at the experimental temperature of 30°, a linear decarboxylation is often obtained lasting over an hour with this organism. Similarly the histidine decarboxylase of *Cl. fallax* Morcom maintains a steady rate at 30°, whereas in most other cases the rate of decarboxylation begins to fall off within 15 min. after mixing. For the experiments involving isolation of the product, quantities of washed suspension etc. were arranged so that the decarboxylation should be complete within an hour and such large scale experiments were checked manometrically.

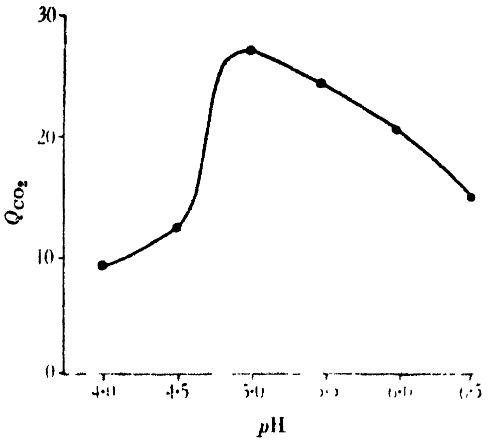


Fig. 3.

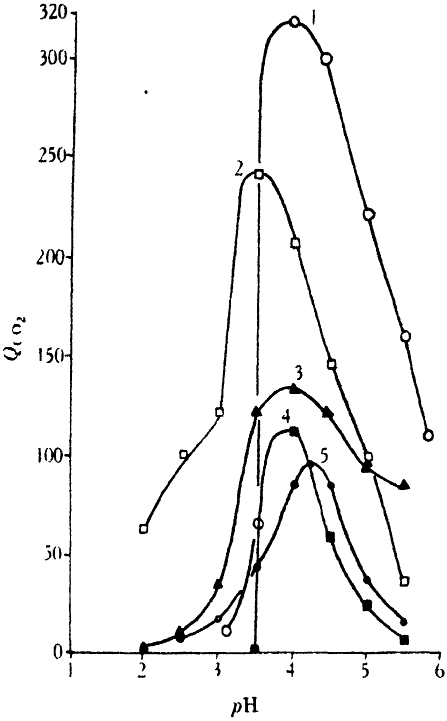


Fig. 4.

Fig. 3. Variation of tyrosine decarboxylase activity (Q_{CO_2}) with *p*H: *Cl. aerofoetidum.*

Fig. 4. Variation of glutamic acid decarboxylase activity (Q_{CO_2}) with *p*H. 1, *Cl. aerofoetidum.* 2, *Cl. welchii* Chelsea. 3, *Cl. welchii* B.W. 21. 4, *Pr. morganii.* 5, *Cl. bifermentans.*

Temperature coefficient. The temperature coefficient has been determined in most cases by carrying out manometric experiments under optimum *p*H conditions and at four different temperatures in each case. Fig. 5 shows the variation of initial Q_{CO_2} in the various cases with thermostat temperature. In most cases a straight line can be drawn through the points obtained for any one enzyme and organism, an exception being the ornithine decarboxylase of *Cl. septique* Pasteur which shows an 'optimum temperature' of 29–30°. There is a general falling-off of activity in most cases for temperatures above 40°. Glutamic acid decarboxylase of *Pr. morganii* shows an unusual variation with temperature, the Q_{CO_2} increasing very rapidly between 25 and 37° and then falling off almost to zero by 40°; only the early portion of this curve is shown on Fig. 5. Table 2 shows the temperature coefficients calculated from the lines shown in Fig. 5.

Effect of oxygen. With one exception the amino-acid decarboxylases of the *Clostridia* are unaffected by the presence of oxygen, the exception being the glutamic acid decarboxylase of *Cl. welchii*, a 50 % inhibition of which is obtained if air is used in the manometers instead of N_2.

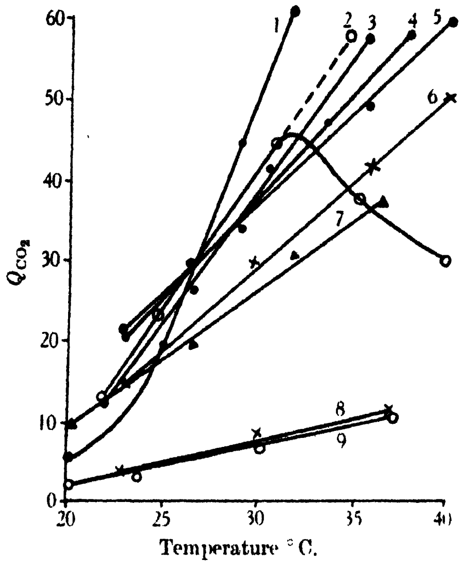


Fig. 5. Variation of activity with temperature. 1, *Pr. morganii*. 2, *Cl. septique* (reduced 5 ×). 3, *Cl. aerofoetidum* (reduced 10 ×). 4, *Cl. bifermentans*. 5, *Cl. welchii* B.W. 21. 6, *Cl. welchii* B.W. 21. 7, *Cl. aerofoetidum*. 8, *Cl. bifermentans*: *Cl. fallax*. 9, *Pr. morganii*.

●—● Glutamic acid. ○—○ Ornithine.
×- - -× Histidine. ▲—▲ Tyrosine.

Table 2. *Temperature coefficients of amino-acid decarboxylases*

Substrate	Organism	Temp. coeff.
Glutamic acid	*Cl. welchii* B.W. 21	1·9
	Cl. aerofoetidum 505	2·7
	Cl. bifermentans 2914	2·0
	Pr. morganii	4·3
Histidine	*Cl. welchii* B.W. 21	2·1
	Cl. bifermentans 2914	2·2
	Cl. fallax Morcom	2·1
Ornithine	*Cl. septique* Pasteur	2·45
	Pr. morganii	2·45
Tyrosine	*Cl. aerofoetidum* 505	2·1

Growth conditions necessary for the formation of the decarboxylases

Age of culture. 2 l. of the 2 % glucose broth medium were incubated in a flask fitted with a three-hole rubber bung: through the bung three glass tubes were passed, one connected to a hydrogen cylinder and conveying the gas below the level of the medium, a second reaching to the bottom of the flask and bent over outside to act as a sampling jet and the third a short tube connected outside to a Bunsen valve, the whole apparatus being assembled under sterile conditions. After inoculating the medium with the contents of a cultured meat-broth tube as usual, a stream of H_2 was swept through the flask to render the contents anaerobic.

Table 3. *Variation of decarboxylase activity with age of culture*

Age of culture hr.	Medium pH	mg. bact./ml. medium	Q_{CO_2}			
			Histidine	Ornithine	Tyrosine	Glutamic acid
Cl. welchii B.W. 21						
0	6·85	—	—	—	—	—
3	6·15	0·181	1·5	—	—	47
4	5·60	0·424	2·5	—	—	107
5	4·83	0·565	4·5	—	—	112
6	4·43	0·755	13	—	—	103
7	4·31	0·830	24	—	—	97
8	4·31	0·890	26	—	—	92
9	4·21	0·960	28	—	—	69
24	4·10	0·965	9	—	—	13
Cl. septique Pasteur III						
0	7·59	—	—	—	—	—
2	7·40	0·030	—	167	—	—
3	7·23	0·081	—	474	—	—
4	6·92	0·134	—	251	—	—
5	6·59	0·185	—	194	—	—
6	6·37	0·226	—	150	—	—
7½	6·03	0·256		166	—	—
8½	5·90	0·270	—	201	—	
10	5·82	0·282		183	—	—
26	5·53	0·311	—	159	—	—
Cl. aerofoetidum 505						
0	7·23	—		—	—	—
3½	7·21	0·061	—	—	33	8·5
4½	6·92	0·122		—	30	7
5½	6·62	0·240	—	—	27	17
7	6·26	0·352	—	—	48	43
8	5·88	0·428	—	—	56	113
9	5·63	0·855	—	—	50	266
10	5·46	0·870	—	—	52	325
26	4·70	0·970	—	—	36	264
Cl. bifermentans 2914						
0	7·04	—	—	—	—	—
3	6·35	0·168	2	—	—	12
4	5·98	0·396	3·5	—	—	92
5	5·79	0·528	7	—	—	125
6½	5·69	0·600	8	—	—	138
7½	5·64	0·660	10	—	—	132
8½	5·58	0·752	8	—	—	131
26	5·22	0·755	5	—	—	94
Cl. fallax Morcom						
0	7·1	—	—	—	—	—
3	6·88	0·048	1	—	—	—
4	6·68	0·165	2	—	—	—
5	6·29	0·333	3·5	—	—	—
6½	5·77	0·480	5	—	—	—
7½	5·47	0·616	5	—	—	—
8½	5·32	0·720	6	—	—	—
9½	5·15	0·800	9	—	—	—
10½	5·12	0·780	12	—	—	—
26	4·63	0·765	6	—	—	—
Pr. morganii						
0	7·19	—	—	—	—	—
2½	6·76	0·061	—	3	—	—
3½	6·55	0·146	—	8	—	—
4½	6·32	0·218	—	16	—	—
6½	5·81	0·346	—	12	—	—
7½	5·70	0·385	—	10	—	—
8½	5·67	0·400	—	7	—	—
26	5·19	0·400	—	6	—	—

In order to take samples at intervals the Bunsen valve was clipped off and the sampling jet opened, when samples of any desired volume could be removed aseptically by slightly increasing the H_2 pressure. In this manner the variation of the decarboxylase activities of the various organisms with 'age of culture' has been followed. The *Clostridia* grow extremely rapidly and turbidity is usually marked 2 hr. after inoculation so that sufficient organisms for the preparation of washed suspensions can be obtained from 300–400 ml. medium after 3 hr. Under these conditions active cell division ceases after 7–11 hr. depending on the organism used, *Cl. bifermentans* 2914 growing the most rapidly of all the organisms tested. With each sample the following determinations were made: *p*H, dry weight of organism present per ml. medium, and decarboxylase activity against the substrates concerned. The results obtained in representative cases are set out in Table 3. They show that, as usual, early cultures display little activity and that the decarboxylases appear at various stages in the growth cycle, reach maximum activity before growth ceases, and then fall off slowly in activity as the culture ages.

It is obvious that the appearance of the decarboxylases is not conditioned only by the age of the culture, as some enzymes, notably the histidine decarboxylase, appear much later in the growth period than others such as the ornithine decarboxylase. Fig. 6 shows the variation of the decarboxylase activity plotted not against the age of the culture but against the *p*H of the medium during growth. From this it appears that the enzymes develop as the *p*H of the medium approaches their optimum *p*H values. Thus it appears that the main factor in this variation of activity with age of culture is due to the variation in medium *p*H during growth. A point of possible theoretical interest is that the medium *p*H at which maximum activity is developed for each enzyme is approximately 1 *p*H unit to the alkaline side of the optimum *p*H for that enzyme. This seems to indicate that the actual *p*H in the vicinity of the enzyme surface within the actively fermenting organism may be below that measured in the medium as a whole.

The case of the ornithine decarboxylase of *Cl. septique* Pasteur is different from the others. A 2 hr. culture has little activity and there is a rapid increase of activity by the 3rd hr. followed by an almost equally rapid fall to a Q_{CO_2} of 150–180 at which level the activity stabilizes for 24 hr. Growth however continues for 8–9 hr. so that in this case there is a marked oscillation of activity during the early phase of growth and it appears on examination of stained films that the organism undergoes a structural change between 3 and 6 hr. The organisms obtained during the first 2·5 hr. stain homogeneously but as growth proceeds organisms appear which stain darkly at their ends and exhibit a granular structure, while spores are first seen at about the 4th hr. The fall in activity after 3 hr. is thus probably related to some structural change in the organism.

Effect of medium p*H during growth.* In the previous studies of amine production by bacteria [Gale, 1940, 1, 2] it has been shown that the amino-acid decarboxylases are produced by the organisms in response to an acid growth environment. That the same is true in the present studies has been indicated by Fig. 6. Flasks have been set up containing broth (without glucose) at adjusted *p*H values, inoculated with the organism, filled with H_2 and then incubated at 37° until growth reached a maximum. The *p*H of the media after growth was measured and the cultures made up into washed suspensions. It was very noticeable in the work with the *Clostridia* that whereas media adjusted before incubation to *p*H 8, 7 or 6 remained approximately at the adjusted values, those adjusted to values below 6 altered considerably during the growth of the organism; thus a medium adjusted to *p*H 5·07 before incubation was found to have *p*H 5·91 on harvesting (*Cl. welchii* B.W. 21). This effect may be due to the greater formation and activity of the glutamic acid decarboxylase at low *p*H values. Fig. 7 shows the amino-acid decarboxylase activity of washed suspensions so prepared plotted against the average *p*H of the medium during growth. The *Clostridia* do not grow very well below *p*H 5·5 in the absence of carbohydrate. The results show, as before, that the enzymes are formed in response to an acid growth environment. In many cases, also as before, the organism growing at *p*H 5 shows as much or greater activity than when grown in glucose broth, showing that the function of the glucose in promoting amine production is to act as a source of fermentation acid. An apparent exception is the histidine decarboxylase of *Cl. welchii*, *Cl. fallax* and *Cl. bifermentans*. In the case of the histidine decarboxylase of *Bact. coli* [Gale, 1940, 1] the enzyme, having an

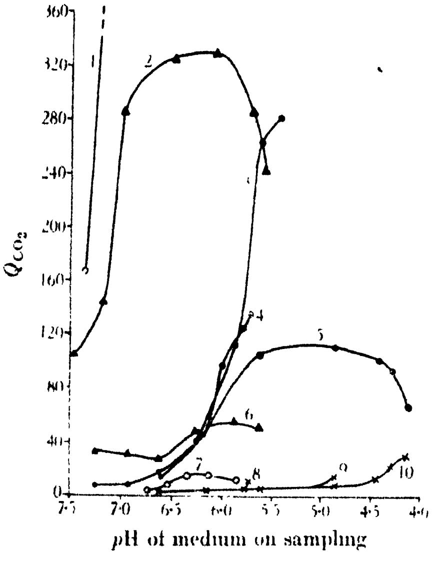


Fig. 6.

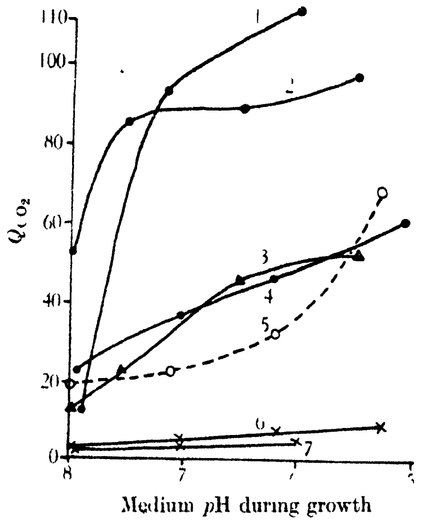


Fig. 7.

Fig. 6. Variation of decarboxylase activity with medium *p*H during growth in glucose broth. 1, *Cl. septique*. 2, *Strep faecalis* [Gale, 1940, 2]. 3 and 6, *Cl. aerofoetidum*. 4 and 8, *Cl. bifermentans*. 5 and 10, *Cl. welchii* B.W. 21. 7, *Pr. morganii*. 9, *Cl. fallax*.

Fig. 7. Effect of growth *p*H on decarboxylase activity of washed suspensions. 1 and 7, *Cl. bifermentans*. 2 and 3, *Cl. aerofoetidum*. 4 and 6, *Cl. welchii* B.W. 21. 5, *Cl. septique* (reduced 10 ×).

●—● Glutamic acid. ○—○ Ornithine.
×—× Histidine. ▲—▲ Tyrosine.

optimum *p*H at 4·0, behaved in this respect like the other decarboxylases, but as a result of the lower *p*H optimum of the *Cl. welchii* histidine decarboxylase, the enzyme in this case requires more acid conditions for its formation than are necessary with *Bact. coli*. Thus in glucose broth the histidine enzyme only appears late in the growth period when the medium *p*H has fallen below 5 (see Fig. 6). When *Cl. welchii* is grown in non-carbohydrate media it is found that the lower the growth *p*H, the more active the decarboxylase, but the activity of a culture grown at *p*H 5 has $Q_{CO_2} = 10$ compared with a value of 60–70 for the same strain

(B.W. 21) grown in the presence of glucose. It is not possible to obtain cultures from non-carbohydrate media at *p*H values much less than 5 so that it appears either that glucose plays a definite part in promoting the formation of this particular enzyme, or that a strongly saccharolytic organism like *Cl. welchii* is able to produce a *p*H in its immediate vicinity during fermentation below that of the medium in general. Whatever may be the reason, it is true to say that *Cl. welchii* will not form the histidine decarboxylase or produce histamine to any extent *in vivo* unless it is actively fermenting carbohydrate.

As has been noted in the case of the ornithine decarboxylase of *Bact. coli*, the ornithine enzyme of *Cl. septique* shows a very marked response to acid growth conditions; the ornithine decarboxylase activity is scaled down 10 times in Fig. 7 in order to compare with the other curves.

Growth temperature. The decarboxylases of *Bact. coli* are formed more actively when the organisms are grown at 27° than at 37°. This does not seem to be the case with either the *Clostridia* or *Proteus*, growth temperature having little effect in most cases while the histidine enzyme is better formed at the higher temperature as, presumably, fermentation occurs more rapidly under such a growth condition.

Isolation and identification of the amines produced

The products of the decarboxylation processes have been isolated in every case from large-scale experiments based on and controlled by manometric work. The manometric experiments indicate in every case that the decarboxylation under optimum conditions proceeds to completion since the theoretical volume of CO_2 is liberated within experimental error. In most cases the entire organisms obtained from 800 ml. glucose broth have been used for each isolation and the concentration of the washed suspension, together with the quantities of buffer and amino-acid, have been arranged so that the decarboxylation should go to completion within 1 hr. The full details of the experimental procedure and isolation are the same as previously described [Gale, 1940, 1, 2]. The yields on isolation of the products of decarboxylation usually amount to 60–70 % of theory. Analyses are by Weiler.

Histidine decarboxylase: product histamine isolated as dipicrate. *Cl. welchii* B.W. 21. Quantities: 20 ml. washed suspension, 15 mg./ml.; 20 ml. 0·1 *M* phthalate buffer *p*H 2·5; 20 ml. *M*/60 *l*(−)-histidine monohydrochloride (neutral). Yield 135 mg.—70 % theory. M.P. 239° (corr.). (Found; C, 35·95; H, 2·67; N, 22·25 %. Calc.: C, 35·97; H, 2·64; N, 22·2 %.)

Similar experiments were carried out with:

Cl. welchii S.R. 9 (*p*H 3·0). (Found: N, 22·08 %.) M.P. 238°. Yield 63 %.

Cl. welchii Chelsea (*p*H 2·5). (Found: N, 22·00 %.) M.P. 241°. Yield 58 %.

Cl. bifermentans 2914 (*p*H 2·5). (Found: C, 36·1; H, 2·68; N, 22·05 %.) M.P. 240°. Yield, 83 %.

Cl. fallax Morcom (*p*H 2·5). (Found: C, 36·2; H, 2·62; N, 21·9 %.) M.P. 240°. Yield, 68 %.

Tyrosine decarboxylase: product tyramine isolated as dibenzoyl deriv. *Cl. aerofoetidum* 505. Quantities: 15 ml. washed suspension 20 mg./ml.; 15 ml. 0·1 *M* phthalate buffer *p*H 5·0; 15 ml. *M*/60 *l*(−)-tyrosine. Yield 65 mg.—74 % theory. M.P. 171°. (Found: C, 76·10; H, 5·47; N, 3·89 %. Calc.: C, 76·50; H, 5·54; N, 4·06 %.)

Ornithine decarboxylase: product putrescine isolated as dipicrate. *Cl. septique* Pasteur III. Quantities: 30 ml. washed suspension 6 mg./ml.; 30 ml. 0·01 *M* phosphate buffer *p*H 5·5; 30 ml. *M*/60 *l*(+)-ornithine dihydrochloride (neutral).

Yield 197 mg.—72 % theory. Decomposes at 250°. (Found: C, 35·69; H, 3·67; N, 20·00 %. Calc.: C, 35·03; H, 3·65; N, 20·44 %.)

Glutamic acid decarboxylase: product γ-aminobutyric acid. Neuberger [1937] states that the dissociation constants of γ-aminobutyric acid are $pK_1 = 4{\cdot}23$ and $pK_2 = 10{\cdot}43$ whereas the author has shown [1940, 1] that the dissociation constants for the α-aminobutyric acid are very different from these, particularly pK_1 which lies in the region of 2. Accordingly it is a simple matter to distinguish between the two possible products of the glutamic acid decarboxylation by carrying out a titration curve on the experimental material and so determining the value of pK_1. The complete isolation has been carried out in the case of *Bact. coli.*

Found: *Cl. welchii* B.W. 21 (pH 4·0). $pK_1 = 4{\cdot}15$.
Cl. aerofoetidum 505 (pH 4·0). $pK_1 = 4{\cdot}25$.
Cl. bifermentans 2914 (pH 4·0). $pK_1 = 4{\cdot}30$.
Pr. morganii (pH 4·0). $pK_1 = 4{\cdot}25$.

Thus the product in all cases of glutamic acid decarboxylation studied has proved to be the γ-aminobutyric acid.

The production of histamine by Cl. welchii in vivo

It has been shown that whenever *Cl. welchii* grows in the presence of fermentable carbohydrate, conditions are such that histamine is produced from free histidine. *Cl. welchii* ferments muscle glycogen with the production of acid and gas almost as rapidly as it attacks glucose, the glycogen fermentation being strictly adaptive. *Cl. welchii* B.W. 21 grown in the presence of glycogen possesses a histidine decarboxylase Q_{CO_2} of 30–50 determined under the usual conditions. Thus it seems probable that when *Cl. welchii* invades muscle tissue conditions arise which are favourable for the production of histamine, as the fermentation of muscle glycogen would produce the necessary low pH, particularly in 'pockets' in such a heterogeneous medium, and the proteolytic activities of the organism would liberate histidine. It seems unlikely that sufficient histamine would be formed under these conditions to produce any symptoms unless in mixed or massive gangrene infections. This aspect of the investigation is dealt with in the Addendum to this paper. Since *Cl. paludis* and the Lamb dysentery organism behave as other *welchii* strains in producing histamine, this may play a part in the aetiology of diarrhoea in lambs.

Inhibitors of histamine production by Cl. welchii

The production of histamine by *Cl. welchii* can be checked either by inhibition of histidine decarboxylase or by inhibition of fermentation during growth. Of a range of substances tested on washed suspensions, $KMnO_4$ and chloramine T prove to be the most active inhibitors, $M/1000$ solutions of either completely inhibiting the histidine decarboxylase and the fermentation of either glucose or glycogen under optimal conditions. $M/1000$ iodoacetate inhibits fermentation reactions but has no effect on histidine decarboxylase. Acriflavin, though very effective as a disinfecting agent as far as *Cl. welchii* is concerned, does not affect the enzymes in question at concentrations less than $M/10$. The decarboxylase is unaffected by concentrations less than $M/100$ of the following: cyanide, urethane, urea, salicylic acid, fluoride, sulphanilic acid, hydrazine, H_2O_2, etc., or by bubbling with O_2.

DISCUSSION

The work presented above gives a general idea of the decarboxylating powers of the *Clostridium* and *Proteus* organisms and the conditions under which such activities can be expected to function. It cannot be regarded as a comprehensive survey as it may well be that some strains of the organisms reported as not showing decarboxylating activities will possess such enzymes, just as only one strain of *Cl. fallax* tested can produce histamine. Those members of the *Clostridium* group which do possess decarboxylases do not appear to have any other marked property in common that distinguishes them from the remainder; they are all strongly saccharolytic, but others of the most saccharolytic of the group, e.g. *Cl. butyricum*, are inactive as far as can be decided at present. *Cl. welchii*, *Cl. septique*, *Cl. bifermentans* and *Cl. aerofoetidum* are all classified in the saccharolytic and proteolytic group of the M.R.C. classification whereas *Cl. fallax* is stated to possess no proteolytic activity. Strains of all these organisms have been reported in the contents of the intestine. Of 9 strains of *Proteus* organisms tested, 7 attacked glutamic acid and 3 ornithine so that the amine-producing activities of this group do not appear to be marked.

SUMMARY

1. Washed suspensions have been prepared of organisms from the groups *Clostridium* and *Proteus* grown in a 2% glucose casein-digest broth and these have been tested for their power to decarboxylate amino-acids.

2. *l*(−)-Histidine is decarboxylated to histamine by strains of *Cl. welchii*, *Cl. bifermentans* and *Cl. fallax*; *l*(−)-tyrosine is decarboxylated to tyramine by *Cl. aerofoetidum*; *l*(+)-ornithine is decarboxylated to putrescine by *Cl. septique* and *l*(+)-glutamic acid is decarboxylated to γ-aminobutyric acid by *Cl. welchii*, *Cl. aerofoetidum* and *Cl. bifermentans*. Of 9 strains of *Proteus* organisms tested, 7 decarboxylated glutamic acid and 3 ornithine.

3. The *p*H optimum for histidine decarboxylase lies between 2·5 and 3·0, for ornithine decarboxylase at 5·5; for tyrosine decarboxylase at 5·0 and for glutamic acid decarboxylase at 4·0.

4. The enzymes appear to vary in thermolability from organism to organism but all have temperature coefficients of the order of 2.

5. When the organisms are grown in glucose broth, early cultures have little activity, the decarboxylases developing later in the growth period as the *p*H of the medium approaches their optimum *p*H. The ornithine decarboxylase of *Cl. septique* is exceptional in that its activity reaches a maximum and then falls again within the first 3 hr. of growth; the loss of activity before growth ceases appears to be associated with a change in structure of the organism.

6. In the absence of carbohydrate the decarboxylase activity of the organism depends upon the *p*H of the medium during growth, low growth *p*H promoting high decarboxylase activity. The histidine decarboxylase activity of *Cl. welchii* is considerably higher when the organism is grown in the presence of fermentable carbohydrate then when it is grown in a non-carbohydrate broth at *p*H 5.

7. The possible pathogenic significance of histamine production by *Cl. welchii* is discussed and methods for its prevention noted.

The author wishes to thank Dr M. Stephenson and Prof. Sir Frederick Hopkins for their interest and encouragement throughout the course of this work. He is indebted to the Royal Commissioners for the Exhibition of 1851 for the Senior Studentship during the tenure of which this work has been carried out.

REFERENCES

Clifton (1940). *J. Bact.* **39**, 485.
Eggerth (1939). *J. Bact.* **37**, 205.
Gale (1940, 1). *Biochem. J.* **34**, 392.
—— (1940, 2). *Biochem. J.* **34**, 846.
—— (1940, 3). *Biochem. J.* **34**, 853.
Kendall & Gebauer (1930). *J. infect. Dis.* **47**, 261.
—— & Schmidt (1926). *J. infect. Dis.* **39**, 250.
Kocholaty & Hoogerheide (1938). *Biochem. J.* **32**, 437.
Neuberger (1937). *Proc. roy. Soc.* A, **158**, 68.
Stickland (1934). *Biochem. J.* **28**, 1746.
—— (1935). *Biochem. J.* **29**, 288, 889.
Woods (1936). *Biochem. J.* **30**, 1934.
—— & Clifton (1937). *Biochem. J.* **31**, 1774.
—— —— (1938). *Biochem. J.* **32**, 345.

ADDENDUM

Plasma histamine in gas gangrene

BY G. L. BROWN, F. C. MacINTOSH AND P. BRUCE WHITE

From the National Institute for Medical Research, Hampstead,
London, N.W. 3

If the symptoms of *Cl. welchii* infection are in part due to histamine intoxication, the histamine concentration of the plasma should be significantly increased. Information about changes in the histamine content of the whole blood is of doubtful value, since, as Code [1937] has shown, most of the blood histamine is in the formed elements. Cat's arterial blood, treated with heparin and centrifuged without delay, yields a plasma whose histamine equivalent may be determined directly upon the atropinized guinea-pig's ileum. Normal cat's plasma, obtained under 'dial' anaesthesia, has a histamine equivalent of not more than 0·01 µg./ml. We have observed that the histamine equivalent of the plasma may be raised to 0·45 µg./ml. by the intravenous injection of 0·5 mg. of histamine per kg. of body weight, or to 0·04 µg./ml. by the subcutaneous injection of the same dose. No obvious symptoms are produced in a cat given 0·5 mg./kg. of histamine subcutaneously.

We have infected cats with a histamine-producing strain of *Cl. welchii* provided by Dr Gale. They were anaesthetized with 'dial', and were maintained under the anaesthesia throughout the experiment. They received an injection into the soleus or gastrocnemius muscle of one leg of 2–6 ml. of a meat-broth culture aged 15–20 hr., and the plasma was examined 2–6 days later. In those animals in which the infection became fully developed, the limb was swollen and crepitant, and the skin covering it discoloured: the muscles of the calf immediately surrounding the site of injection were broken down to a pulpy mass, and in certain instances there was a considerable collection of thick creamy pus which yielded only *Cl. welchii* on culture. Some of the animals died, but the cat appears able to withstand an extensive involvement of one limb without succumbing to the effects of toxaemia. In no instance could *Cl. welchii* be recovered from the blood.

The histamine equivalent of the plasma from the infected animals has in no case been greater than 0·02 μg./ml., and in two animals in which the infection was advanced and the assay particularly satisfactory, values of 0·008 and 0·007 μg./ml. were obtained. We took the occasion, in four animals, to determine the histamine equivalent of normal and infected muscles. The histamine equivalent of the infected muscles was greater, by 30 to 230 %. If the normal histamine content of cat's muscle is taken to be 1 μg./g., it can be calculated that at least 300 g. of infected tissue would be required to yield 1 mg. of histamine. The histamine equivalent of the purulent exudate in one animal was about the same as that of normal muscle.

Clinical experience shows, and we have found in the cat, that an infection with *Cl. welchii* proceeds more rapidly if other anaerobes such as *Cl. sporogenes* or *Cl. histolyticum* are present. These organisms, although not themselves histamine producers, are actively proteolytic, and might be expected, by increasing the amount of histidine available, to accelerate the liberation of histamine by *Cl. welchii*. In a cat which had received a mixture of 20 hr. broth cultures of *Cl. welchii* (1 ml.) and *Cl. histolyticum* (0·5 ml.), the infection was well developed in 28 hr., involving the thigh muscles as well as the gastrocnemius. The histamine equivalent of the plasma was less than 0·005 μg./ml. The histamine equivalent of the infected and pultaceous muscle was about 30 % higher than that of the normal side.

It would appear, therefore, that in cats infected with *Cl. welchii*, no significant pathological effect is produced by the absorption of histamine into the blood stream from the infected tissue.

REFERENCE

Code (1937). *J. Physiol.* **90**, 349.

II. THE COLORIMETRIC ESTIMATION OF URIC ACID IN FOWL'S EXCRETA

By JOHN CHARLES DUNCAN HUTCHINSON

Department of Poultry Nutrition, Animal Nutrition Institute, School of Agriculture, Cambridge

(*Received 30 November 1940*)

NUMEROUS methods have been elaborated for the estimation of uric acid in avian excreta. These methods and their defects have been admirably reviewed by Fritz [1935]. In this laboratory a modification of Woodman's [1924] method has been in use, in which the nitrogen in the precipitate obtained is estimated by the Kjeldahl method. It is found that this nitrogen is about 35 % less than would correspond to the weight of the precipitate if the latter were pure uric acid. It has been assumed that the impurities present in the precipitate contain no nitrogen and if this is the case the method may be accurate, but if nitrogenous impurities are present the results must be too high. Fritz [1935] has found that uric acid is liable to be destroyed by extraction with lithia, and Folin & Denis [1912] have called attention to the fact that uric acid is very rapidly destroyed on boiling in alkaline solution. The results may be too low on this account. In addition, the method is very laborious, owing to the filtration difficulties and the numerous processes involved. Consequently it is not very practical for metabolic work in which it is necessary to make a large number of estimations and to obtain the results quickly.

St John & Johnson [1931] have worked out a more refined isolation technique in which piperidine replaces lithia as the solvent for uric acid. This method appears to be reliable, but from the results published in their paper it seems that the quantity of nitrogen found in their precipitate averages 2·7 % less than corresponds with the weight of precipitate. There is thus still a small error caused by lack of specificity. If there is any nitrogenous impurity in the precipitate the error may be greater than 2·7 %. If on the other hand the uric acid content is calculated from a nitrogen estimation on the precipitate to eliminate the error, the method becomes nearly as cumbrous as Woodman's procedure, and in any case a more rapid method is desirable. Fritz [1935] also states that loss of uric acid is very liable to occur in piperidine solution when this method is employed.

The author has tried to separate uric acid added to human faeces by the methods of St John & Johnson [1931] and of Woodman [1924]. It was not possible to get a full recovery, because the filtration processes under these conditions are very lengthy and loss of uric acid occurred in alkaline solution, but it was found that by St John & Johnson's method the nitrogen obtained from the precipitate was 21 % less than corresponded to the weight of precipitate, while by Woodman's method it was 34 % less. There was no evidence of nitrogenous impurities in the precipitate obtained by St John & Johnson's method, but nitrogenous impurities were present in the precipitate obtained by Woodman's method.

The colorimetric method is much less laborious than the isolation methods, and it has been developed to a high degree of specificity for estimations in blood and urine, chiefly by Benedict and by Folin. Preliminary experiments were made by the author, using the Benedict & Franke [1922] method for urine, on phosphate and lithia extracts of dried sheep's and human faeces to which 7 % of uric acid had been added. The uric acid recovered exceeded by 4·3 % the amount added to sheep's faeces and by 3·3 % in the case of human faeces. The extracts were not sufficiently dark coloured to affect the colorimeter readings. It was clear from these experiments that the colorimetric method was highly specific for uric acid in faecal extracts and that it would only be necessary to introduce one stage of purification to obtain an accurate method.

The method described in this paper has been worked out for use with Benedict's [1922] arsenophosphotungstic acid reagent and Newton's [1937] lithium arsenotungstate reagent. A great deal of work has been published on methods of intensifying the blue colour. Christman & Ravwitch [1932] found no true proportionality of colour intensity to the concentration of uric acid when Benedict's [1922] 5 % cyanide solution was used. Moreover, it varied with different samples of cyanide and also was less reliable for diluted urine than for pure uric acid solutions. They found that they could correct the proportionality by adding 10 % of sodium carbonate to the cyanide solution and claimed that this solution gave a higher degree of specificity to the colour reaction. They considered that the variation in different samples of cyanide was due to their different contents of carbonate. In the author's experience however the proportionality is not the same with different samples of cyanide of similar origin used for making up Christman's solution, so that it seems that some other factor besides the carbonate content is involved. The author has compared the proportionality observed when Christman's solution was used with that observed when Folin's [1933] cyanide urea solution (which is free from carbonate) was employed. Both solutions were prepared from the same sample of sodium cyanide (B.D.H. pure). Uric acid standards containing 0·2, 0·3 and 0·4 mg. per 10 ml. were prepared by dissolving pure uric acid in warm lithium carbonate and adding acid lithium chloride, as described later, to prevent turbidity from developing during the colour reaction. 10 ml. of the standard solution, 5 ml. of cyanide and 2 ml. of undiluted Newton's reagent were used to develop the colour. On comparing the 0·2 and 0·4 mg. standards with the normal standard of 0·3 mg., the proportional colour intensity was found to increase about 5 % over this range as the concentration of uric acid was increased when Christman's solution was used. That is, the 0·4 mg. standard was 2·5 % too high and the 0·2 mg. standard was 2·5 % too low. When Folin's cyanide urea solution was employed the proportional colour intensity decreased 6 %. Thus the effect of adding the carbonate was to reverse the error in proportionality. It decreased the total intensity of the colour as Folin states. It seems that there should be an optimum quantity of carbonate which will give true proportionality with any given sample of cyanide, though this quantity varies with different samples. For example, a second sample of cyanide (also B.D.H. pure) gave true proportionality with Newton's reagent and Christman's cyanide. With a third sample (B.D.H. pure) the proportional colour intensity increased 5·5 %, practically the same amount as with the first sample.

Similar results were obtained with 2 ml. of the Benedict & Franke [1922] reagent. With the first sample of cyanide the proportional colour intensity increased 7 %, the second gave true proportionality, while the third gave an increase of 5 %.

A large number of experiments on proportionality were carried out with extracts of fowl's excreta both by the direct and indirect methods described later. In contrast to the experience of Christman & Ravwitch [1932] with diluted human urine, the proportionality was found to be exactly the same with extracts of excreta as with lithium carbonate solutions of uric acid.

When an ammoniacal precipitating solution is used for the uric acid, the residual ammonia remaining in the precipitate can depress the colour reaction. This depressant effect is considerably greater for cyanide solutions with high driving power, such as Benedict's [1922] and Folin's [1933], than for Christman's solution. When the Benedict & Hitchcock [1915] 'ammoniacal silver magnesium precipitating solution' is used, as in the method described below, there appears to be an advantage in using Christman's solution for the colour development. Moreover, Christman & Ravwitch [1932] claim that their solution is more specific for uric acid than a 5 % solution of sodium cyanide without the addition of carbonate. Folin [1934], on the other hand, considers that increasing the driving power by removal of traces of carbonate increases the specificity and therefore uses cyanide solutions in which the carbonate is completely removed. For the estimation of uric acid in fowl's excreta no marked difference has been observed as regards the specificity of Folin's and Christman's solutions, but if ammonia can affect the action of Folin's solution more than that of Christman's solution, it may be that other extraneous factors can also affect it more than Christman's solution under practical conditions. For this work therefore Christman's solution has been used as the standard reagent. It has been confirmed that its driving power remains unimpaired for several months.

The preliminary experiments reported above on faecal extracts showed that to obtain the necessary specificity it was desirable to separate the uric acid from the extract prior to developing the colour. For the precipitation of uric acid the zinc hydroxide method of Morris & Macleod [1922] and the Salkowski reaction as employed by Benedict & Hitchcock [1915] have been tried. The zinc method appeared to bring down a considerable quantity of colouring matter as well as the uric acid. Moreover, the zinc was found to exert a depressant effect on the colour reaction, as Kern & Stransky [1937] had also observed, so that only 97 % recovery could be obtained from lithium carbonate solutions of uric acid. The results by this method, after correcting for the loss in the recovery, were practically the same as with the Benedict & Hitchcock [1915] method, in spite of the lack of specificity. Benedict and his co-workers consider that the Salkowski reaction provides the most specific method of precipitating uric acid, and it has therefore been adopted as the standard method. Here again difficulty was experienced in getting a full recovery of uric acid from lithium carbonate solution. This is partly because the reaction is not absolutely quantitative [Folin & Wu, 1919], and partly because the residual ammonia present in the precipitate can depress the colour reaction. The best results were obtained by using the proportions of precipitant recommended by Benedict & Hitchcock [1915], namely, 1 ml. precipitant to 5 ml. extract, and not 5 ml. precipitant to 5 ml. extract as used by Christman & Ravwitch [1932]. If the neck of the centrifuge tube is swabbed out carefully after draining and a current of air is passed over the precipitate, most of the residual ammonia can be eliminated so as not to interfere with the colour reaction. By this means a recovery of 98·5 % was obtained and the results were corrected accordingly. This correction may vary up to 1 % with individual workers and should be determined individually. The amount of ammonia remaining in the silver magnesium urate precipitate after passing the current of air should not exceed 1 ml. $N/10$.

To decompose the precipitate and remove the silver, which may interfere with the colour reaction, 7 % lithium chloride in 0·125 N HCl was used in place of Folin's acid NaCl procedure. The advantage in the use of lithium chloride is that it prevents the development of turbidity during the colour reaction. It was found that if lithium chloride was not present the urea in the cyanide solution was barely sufficient to prevent turbidity when Benedict's reagent was used, and definitely insufficient with Newton's reagent. Benedict & Behre [1931] have also used an acid lithium chloride solution for this reason. The decomposition of the precipitate by acid chloride not only removes the silver, but has the advantage of bringing down most of the colouring matter which may be present, so that a colourless solution of uric acid is obtained even from the most deeply coloured extracts of excreta. It is found that no uric acid is lost by this procedure, even though the concentration of uric acid approximates to, or in some cases even exceeds, its solubility in acid solution.

The author, like Christman, has not found Benedict & Hitchcock's [1915] phosphate standard uric acid solution to be reliable. Folin's [1930] most recent formalin standard has proved to have excellent keeping qualities. On dilution it was found to be exactly equivalent in colour-producing property to a lithium carbonate solution of uric acid of the same strength. A diluted solution of this standard containing 0·3 mg. uric acid in 10 ml. is prepared for use and this will keep for 14 days. A quantity of acid lithium chloride solution equal to that present in the unknown must be added to the standard, because chloride intensifies the colour reaction, at all events when Benedict's arsenophosphotungstic acid reagent is employed.

Fritz [1935] found that loss of uric acid is very liable to occur when it is extracted from the excreta by lithia or piperidine. The author has found that piperidine depresses the colour reaction. Phosphate is not suitable because it interferes with the precipitation of uric acid, causing turbidity in the subsequent colour reaction, though it gives only a lightly coloured extract. Dilute lithium carbonate has been successfully employed by Yamaguchi [1930] and by Furth & Edel [1933] for the extraction of uric acid from tissues. It has proved very successful for extracting the uric acid from fowl's excreta in the method described below.

Woodman [1924] considered it desirable to convert any ammonium urate present in the excreta into uric acid with HCl before extracting with lithia, since unless this were done the extraction of uric acid was apparently not complete. St John & Johnson [1931] also adopt this procedure before their piperidine extraction. Some experiments were made to find out whether this preliminary treatment was necessary in the case of fowl's excreta. Samples were estimated by the standard colorimetric technique described below, with and without pretreatment with HCl. 0·8 g. of excreta was covered with 7 ml. of distilled water and 7 ml. 0·3*N* HCl were added. The mixture was brought to boiling point and held there for 4 min. In the first two experiments enough lithium carbonate was added to neutralize the HCl in addition to the 12 ml. normally used for the extraction. In the other two experiments the mixture was left for 24 hr. after boiling with HCl and then filtered and washed once with distilled water. The residual HCl was neutralized with lithium carbonate and extracted in the same way. The results of these experiments were as follows (Table 1).

Table 1

	% uric acid on air-dried wt.	
No. of exp.	With HCl pretreatment	Without HCl pretreatment
1	8·49	8·43
2	8·57	8·60
3	10·13	10·32
4	9·25	9·10

It is apparent that preliminary treatment with HCl is unnecessary.

The following standard procedure has been adopted for the estimation of uric acid in avian excreta. Since eight estimations can be performed in one day, there is a considerable saving of labour as compared with the older methods.

Method

1. Enough air-dried excreta to give 50–100 mg. uric acid is weighed and washed into a 250 ml. volumetric flask with 20 ml. of distilled water.
2. 12 ml. 0·5 % lithium carbonate solution or, if more than 0·8 g. excreta were taken, 15 ml., are added. The flask is heated on a water bath for 15 min., cooled immediately and made up to volume.
3. The extract is filtered, the first few ml. being rejected if there is any turbidity.
4. 5 ml. filtered extract are pipetted into a 25 ml. centrifuge tube and 1 ml. of the Benedict & Hitchcock [1915] 'ammoniacal silver magnesium precipitant' is added. The liquid is stirred very carefully with a fine glass rod so that it does not splash up the sides of the tube and is then centrifuged for 5 min.
5. The supernatant liquid is poured off and the tube is drained for 2 min. Its neck is then swabbed with a filter paper. The tube is placed in a beaker of hot water (70–80°) and a current of air is passed for 1 min. over the precipitate to remove ammonia. The precipitate is dislodged from the bottom of the tube by a smart tap, the tube is replaced in the beaker of hot water and the current of air is passed for another minute.
6. 20 ml. 7 % lithium chloride solution in 0·125 N HCl are added and the contents of the tube are stirred very thoroughly with a fine glass rod until all the precipitate is decomposed. The tube is then centrifuged for 2–3 min.
7. The supernatant liquid is poured into a 50 ml. volumetric flask, 5 ml. of the acid lithium chloride solution are added to the centrifuge tube and 10 ml. distilled water. The contents are stirred and centrifuged again for 2–3 min.
8. The supernatant liquid is poured off into the 50 ml. flask and the liquid is made up to volume with distilled water.
9. The standard and unknown are prepared simultaneously for the colour development. 10 ml. of the solution in the 50 ml. flask and of the diluted standard are pipetted each into 50 ml. volumetric flasks. 5 ml. of the Christman's cyanide solution are added and either 2 ml. arsenophosphotungstic acid reagent or 1·5 ml. Newton's lithium arsenotungstate reagent undiluted; the flasks are shaken gently. The colour is allowed to develop for 5 min. with Benedict's reagent or 8 min. with Newton's reagent, and the mixtures are made up to volume with distilled water.
10. The colours are compared in a colorimeter immediately. Readings between 15 and 30 mm. are satisfactory provided that the reagents are adjusted to give true proportionality as described above. The results are corrected for the empirical loss in the recovery of uric acid after precipitation.

Recovery of uric acid from fowl's excreta

The following experiment was performed to test the recovery of different quantities of uric acid from fowl's excreta. A sample of excreta was separated mechanically as far as possible from uric acid and dried. Different quantities of uric acid were added to this, and the uric acid was estimated by the standard colorimetric method described above, using Benedict's colorimetric reagent

The results are given in Fig. 1. The amounts of uric acid added were recovered quantitatively over the whole range of conditions likely to be encountered in practice, from the low uric acid content of excreta from birds on a nitrogen-free ration to the high content which is obtained when they are on a nitrogen-rich ration. It appears that the amount of protein which is present in the excreta is not sufficient to affect the precipitation at low concentrations of uric acid. The graph (Fig. 1) does not run through the origin because there was a small quantity of residual uric acid in the excreta after the mechanical separation.

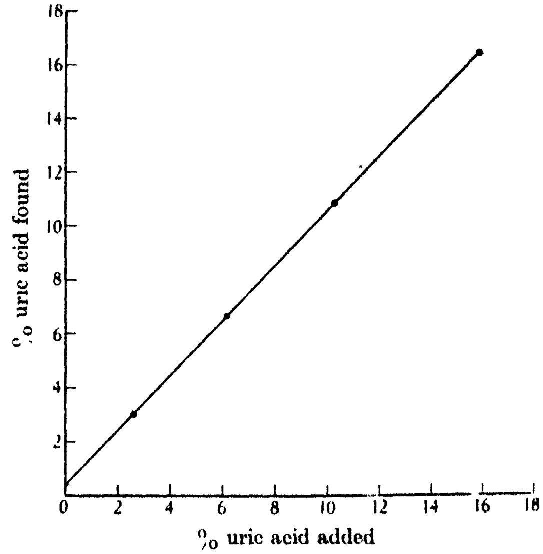


Fig. 1. Recovery of uric acid added to 'nearly uric acid-free' fowl's excreta.

Specificity

To test the specificity of the method it was necessary to add the uric acid to sheep's and human faeces because it was impossible to obtain fowl's excreta completely free from uric acid. A lithium carbonate extract was prepared from dried sheep's faeces, and a uric acid solution in lithium carbonate was added to this to provide the equivalent of 7·5% of uric acid reckoned on the air-dried weight of the faeces. Thus, any error in the result could not be caused by failure of the extraction process, but must be due to lack of specificity. The uric acid content of this mixture was then estimated by the standard method, using Benedict's colorimetric reagent. The results were as follows (Table 2):

Table 2

No. of exp.	% uric acid added	% uric acid recovered
1	7·50	7·55
2	7·50	7·58
3	7·50	7·58
4	7·50	7·53
Mean	7·50	7·56

Further experiments were performed on sheep's and human faeces. Ammonium urate instead of uric acid was added before extraction and Newton's colorimetric reagent was used. The results are given in Table 3.

Table 3

Source of faeces	Weight of air-dried faeces g.	Ammonium urate added g.	Ammonium urate recovered g.
Sheep	0·700	0·0805	0·0810
„	0·748	0·0825	0·0800
„	1·331	0·0835	0·0801
„	1·400	0·0815	0·0829
„	1·403	0·0830	0·0840
Human	0·754	0·0825	0·0833
„	1·503	0·0910	0·0946

These tests show that the method is highly specific and gives good results under conditions which caused a failure of the methods of Woodman [1924] and of St John & Johnson [1931] (see p. 81).

Experiments were also carried out to test the specificity of the Benedict and Newton colour reagents in the presence of certain other purine bodies which are also precipitated by the ammoniacal silver magnesium precipitant. Solutions or suspensions were made up in lithium carbonate of guanine, adenine, hypoxanthine and xanthine containing 0·75 mg. of the purine bodies and 0·3 mg. of uric acid per 10 ml. of solution. The colours produced by 10 ml. of these solutions were compared with that produced by 10 ml. of a standard uric acid solution (0·3 mg. per 10 ml.). Adenine and hypoxanthine gave no colour with either reagent. Xanthine gave 1/40th of the colour which would be given by the same quantity of uric acid when Newton's reagent was used for the colour reaction, and 1/30th of the colour with Benedict's reagent. Guanine gave 1/70th of the colour with Newton's reagent and 1/25th of the colour with Benedict's reagent. Thus the small quantity of the other purine bodies precipitated with uric acid could not affect the results. The Newton reagent appears to be rather more specific than the Benedict reagent, as Newton states. Fürth & Edel [1933], using Folin's colorimetric reagent, have also found that the decomposition products of the nucleic acids are either not precipitated or give no colour.

The possibility of using a direct method for the estimation of uric acid in avian excreta has been examined. Estimations made directly on the lithium carbonate extracts of the excreta have been compared with those made on the same extracts by the standard indirect method described above. Both Newton's and Benedict's colorimetric reagents were used in these determinations, and some experiments were made using Folin's carbonate-free cyanide urea solution. For the direct estimations with Christman's cyanide solution the lithium carbonate extracts were diluted 5 times to give a convenient concentration of uric acid, and the same amount of acid lithium chloride was added as was contained in the standard. For the direct estimations with Folin's cyanide-urea solution a standard was prepared containing 0·06 mg. uric acid per 10 ml. and the extracts of the excreta were diluted 25 times. To develop the colour when Folin's cyanide solution was used, 10 ml. diluted extract were taken, 5 ml. cyanide-urea solution and 1 ml. undiluted Newton's reagent were added. The volume was made up to 20 ml. after 10 min. The results of these experiments are given in Table 4.

The specificity of Newton's reagent combined with Christman's cyanide solution is high when used directly on the lithium carbonate extract. The results obtained by this method were on the average 1·8 % higher than those obtained by the indirect method. The Benedict reagent was not quite so specific, yielding results which averaged 3·9 % above those obtained by the indirect method. The experiments in which the Newton reagent was combined with Folin's cyanide-urea solution gave values which were intermediate between those of the other two direct methods, being 2·6 % above those of the indirect method. Samples 5, 6, 7 and 8 were used for comparing the Newton and the Benedict reagents in the indirect method in conjunction with Christman's solution. The results for these samples are not the same for the two series, because they were part of a series of estimations carried out at the extremes of the range of uric acid concentrations to test the proportionality of the colour to the uric acid concentration.

Table 4

	Direct method		Indirect method	
No. of sample	% uric acid on air-dried wt.	Colorimetric technique	% uric acid on air-dried wt.	Colorimetric technique
1	12·68	N C	12·51	N C
2	11·21	N C	11·11	N C
3	8·88	N C	8·55	N C
4	10·30	N C	9·96	N C
5	10·77	N C	10·76	Mean of B C & N C
6	9·37	N C	9·43	Mean of B C & N C
7	8·04	N C	7·67	Mean of B C & N C
8	10·02	N C	9·96	Mean of B C & N C
13	3·16	N C	3·01	Mean of B C & N C
5	11·10	B C	10·60	Mean of B C & N C
6	9·21	B C	9·04	Mean of B C & N C
7	8·84	B C	8·53	Mean of B C & N C
8	11·15	B C	10·41	Mean of B C & N C
13	3·11	B C	3·04	Mean of B C & N C
9	10·02	N F	9·72	B C
10	7·11	N F	6·86	B C
11	6·75	N F	6·42	B C
12	8·30	N F	8·24	B C
13	3·04	N F	3·03	Mean of B C & N C

N = Newton's coldrimetric reagent.
B = Benedict's colorimetric reagent.
C = Christman & Ravwitch's cyanide carbonate urea solution.
F = Folin's carbonate-free cyanide urea solution.

While under the conditions employed the proportionality was not exact, it was practically the same for the two colorimetric reagents. When Newton's reagent combined with either Christman's or Folin's cyanide solution is used directly on the extracts the method is at least as specific as that of St John & Johnson [1931]. It seems that the rather heroic efforts of Folin and Benedict and their co-workers to prepare a direct uric acid colorimetric reagent, if not altogether successful for blood estimations, have at least produced a technique of practical value for direct determinations on avian excreta. On the other hand, the indirect method is more reliable and should be employed as the standard procedure. Where, however, saving of time is important, and it is known that the direct and indirect methods agree in the estimation of uric acid in excreta obtained from birds on a ration of the same type as that under investigation, the direct method may be of considerable practical value. Close agreement between direct and indirect methods has been obtained also by Shannon [1938], working on fowl's urine which has been separated from the faeces.

Experiments have been carried out to test whether there is any difference between the results obtained in the indirect method when either Benedict's or Newton's reagent is combined with Christman's cyanide solution. The results are given in Table 5.

Table 5

No. of sample	% uric acid on air-dried wt. Benedict's reagent	 Newton's reagent
5	10·74	10·63
6	9·22	9·26
7	8·00	8·21
8	10·29	10·08

There was no significant difference between the results obtained by the two methods. In general it was found that Newton's reagent with Christman's cyanide solution did not give such close agreement between duplicates as the Benedict and Franke reagent.

Finally, a number of samples of fowl's excreta have been analysed by the author and other workers in this laboratory by the modification of Woodman's method used here hitherto: the results were compared with those obtained by the standard indirect colorimetric procedure and are given in Table 6.

Table 6

No. of sample	% uric acid on air-dried wt. Colorimetric method	 Woodman's method
18	9·61	8·98
19	8·46	9·04
20	9·92	8·66
21	7·35	6·48
22	8·20	7 90
23	8·19	7 17
24	9·78	7 18
25	6·84	6·04
26	6·51	6 03

The results by Woodman's method are in most instances considerably lower than those obtained by the colorimetric method. This is presumably due to the fact that the destruction of uric acid brought about by boiling with lithia more than compensates for any increase in the apparent value caused by nitrogenous impurities which may be present in the uric acid precipitate.

SUMMARY

A colorimetric method is described for the determination of uric acid in fowl's excreta; it is both more specific and less laborious than the methods at present in use.

The author is indebted to Mr E. T. Halnan and Mr F. W. Foreman for able criticism during the course of the work.

REFERENCES

Benedict (1922). *J. biol. Chem.* **51**, 187.
—— & Behre (1931). *J. biol. Chem.* **92**, 161.
—— & Franke (1922). *J. biol. Chem.* **52**, 387.
—— & Hitchcock (1915). *J. biol. Chem.* **20**, 619.
Christman & Ravwitch (1932). *J. biol. Chem.* **95**, 115.
Folin (1930). *J. biol. Chem.* **86**. 179.
—— (1933). *J. biol. Chem.* **101**, 114.
—— (1934). *J. biol. Chem.* **106**, 311.
—— & Denis (1912). *J. biol. Chem.* **13**, 469.
—— & Wu (1919). *J. biol. Chem.* **38**, 81.
Fritz (1935). *J. industr. Engng Chem.* **7**, 123.
Fürth & Edel (1933). *Biochem. Z.* **263**, 9.
Kern & Stransky (1937). *Biochem. Z.* **290**, 419.
Morris & Macleod (1922). *J. biol. Chem.* **50**, 55.
Newton (1937). *J. biol. Chem.* **120**, 315.
St John & Johnson (1931). *J. biol. Chem.* **92**, 41.
Shannon (1938). *J. cell. comp. Physiol.* **11**, 135.
Woodman (1924). *J. agric. Sci.* **14**, 413.
Yamaguchi (1930). *Jap. J. med. Sci.* IV, Pharmacology, **5**, 72.

12. SEPARATION OF THE HIGHER MONOAMINO-ACIDS BY COUNTER-CURRENT LIQUID-LIQUID EXTRACTION: THE AMINO-ACID COMPOSITION OF WOOL

By A. J. P. MARTIN and R. L. M. SYNGE[1]

From the Wool Industries Research Association, Torridon,
Headingley, Leeds[2]

(*Received 31 December 1940*)

Introduction

THE possibility of employing counter-current liquid-liquid extraction for the separation of amino-acids in the form of acyl derivatives from protein hydrolysates was briefly discussed by Synge [1939, 1–4], who described preliminary experiments on the separation of some of the naturally occurring 'monoamino-acids'. In the present paper we review the literature on counter-current liquid-liquid extraction, discuss the mathematical and physical basis of the separation of acylamino-acids by liquid-liquid extraction, and describe the construction, operation and testing of a multi-plate chloroform-water counter-current extraction train. We then describe the use of this machine in a control analysis of a known mixture of amino-acids for such components as methionine, valine, proline, the leucines and phenylalanine, together with the application of the new technique to an analysis of wool for these amino-acids. In conclusion we compare the results with those obtained by other methods, and summarize existing knowledge of the amino-acid composition of wool.

Historical

The development of a mathematical treatment of the problems of continuous fractional distillation has made clear the analogy that exists between all counter-current processes. Graphical methods are generally employed, two of the most important being those of Ponchon [1921; cf. Thiele, 1935] and of McCabe & Thiele [1925]. Randall & Longtin [1938, 1–3; 1939, 1–3] have pointed out that the McCabe-Thiele diagram is a point-line contact transformation of the Ponchon diagram, which latter is for many purposes more convenient. These authors have generalized the method of Ponchon for dealing with counter-current processes of all kinds, including liquid-liquid extraction.

Although extraction by immiscible solvents has long been one of the standard purification techniques of the organic chemist, the advantages offered by the application of counter-current principles to this process are only at the present day beginning to be recognized. Nearly all development in this field has so far been in connexion with industrial processes.

---

[1] International Wool Secretariat Student.

[2] The preliminary stages of the work described in this paper were carried out in the Dunn Biochemical and Nutritional Laboratories, Cambridge.

The advantages of this technique, as against distillation, are chiefly these:

(1) Non-volatile and thermolabile materials may be fractionated.

(2) Very small quantities of material may be fractionated by employing dilute solutions. In distillation this is impossible unless a suitable 'carrier' substance is available.

(3) In extractional fractionation partition coefficients—a function of the balance of polar and non-polar groups in the molecule—are the determining property. In distillation, volatility determines the separation. This renders, in many cases, fewer theoretical plates necessary for extractional than for distillatory separation.

Lewis [1916] discussed the application of counter-current principles to the extraction of solids and gases by liquids. Hunter & Nash [1932] applied the McCabe-Thiele diagram to counter-current liquid-liquid extraction. The analogy between this process and distillation was also recognized by Saal & van Dijck [1933], who applied the method of Ponchon to the usual triangular phase diagram for ternary systems. This was also done by Hunter & Nash [1934]. Evans [1934] and Thiele [1935] have discussed the analogy between distillation and extraction, and reviewed previous theoretical treatments of extraction processes. Thiele points out the analogy between extraction columns with successive mixing and settling chambers and plate distilling columns, and shows how continuous-contacting extraction columns (packed or spray types) can be treated by making use of the H.E.T.P. concept, as for packed distilling columns.

During this period counter-current liquid-liquid extraction has become of great importance in the refining of lubricating oils; extraction by liquid sulphur dioxide, nitrobenzene and cresylic acid-liquid propane ('duo-sol' process) have been employed. The efficiency of packed and spray towers has been accurately determined (cf. 'Symposium on Absorption and Extraction', *J. industr. Engng Chem.* **29** (1937), and elsewhere in the same journal).

So far the industrial applications of liquid-liquid extraction have not necessitated columns of very high 'plate number', and in most cases spray or packed columns (which have much greater H.E.T.P. than when used for distillation), or a few mixing and settling units in series have been employed.

The only published work describing a 'multi-plate' column suitable for laboratory use is by Cornish *et al.* [1934], who constructed a counter-current column of 210 units, each with a plate efficiency of about 50% under the conditions of operation. The apparatus had alternate stirring and settling chambers, and was made of nickel. It was used for the fractionation of fat-soluble vitamins between such solvents as octane and methyl alcohol. The procedure employed was to inject the material to be fractionated at the middle of the column at the beginning of the run, and at the end of the run to collect the two fractions that had flowed out at either end and that which was left in the column.

This procedure differs from all the industrial processes described above, which are essentially continuous. Batch operation necessitates a different theoretical treatment, and Cornish and co-workers obtained an expression for the proportion of material left in the column after a given time of running where the number of theoretical plates, rates of liquid flow and volume of solvents per theoretical plate are given, where the solute is distributed between the two phases according to Henry's distribution law, and where the flow ratio of the two solvents is the inverse of the partition coefficient of the solute under consideration. Under these circumstances the movement of the solute is equally towards each end of the column, and is directly analogous to the flow of heat along a bar.

In connexion with the present work, where a similar mode of operation was adopted, Daniels & Martin, in a forthcoming paper, have generalized and extended the theoretical treatment to deal with solutes obeying Henry's law, but whose partition coefficients differ from the inverse flow ratio of the solvents. They have incidentally shown that the distribution of solute that emerges at opposite ends of the column after an infinite time of running is the same as that which would be obtained under conditions of continuous operation, as calculated from the McCabe-Thiele or similar diagram.

One of us (A. J. P. M.) (unpublished work carried out at the Dunn Nutritional Laboratory, Cambridge) has constructed a glass multi-plate column working with the phases of a petroleum-methyl alcohol mixture, and has applied it to the purification of vitamin E.

Physical chemistry of the separation

In the present work we have separated acetamino-acids by partition between chloroform and water phases. The reasons for using the acetyl derivatives, and some data on their distribution between immiscible solvents, have been given by Synge [1939, 1, 2]. We have used the solvent pair chloroform-water because of the considerable differences between the partition coefficients of acetamino-acids given by these solvents. In addition, the absolute values of these partition coefficients (which determine the flow ratios employed in the column) lie in a suitable range. This is not the case for carbon tetrachloride and water, between which solvents *N*-acetyl-*dl*-leucine has $P=130$ ($37°$, $c=3{\cdot}2$).

Here and subsequently,

c = concentration of solute in aqueous phase, expressed as mg. per ml.,

$$P=\frac{\text{Concentration of solute in aqueous phase}}{\text{Concentration of solute in organic solvent phase}}.$$

An investigation of the distribution of acetamino-acids between petroleum-methyl alcohol phases showed very little 'spread' of the partition coefficients, and these had a very high temperature coefficient. It appeared that the acetamino-acid distribution followed very closely the distribution of methyl alcohol between the phases.

The use of chloroform-water had other secondary advantages: the phases separate readily, having a large difference in density and a high surface tension. In addition, B.P. chloroform, containing a trace of alcohol, does not readily undergo decomposition; we have never detected any liberation of Cl^- into the aqueous phase. Chloroform is non-inflammable. The operation of the column is not affected by atmospheric moisture, as when an anhydrous solvent pair is employed.

In view of the considerable temperature coefficient of the partition coefficients, it was necessary to maintain the column at a constant temperature. The highest practicable working temperature (37°) was employed, as this renders the acetamino-acids more readily extractable into chloroform, and thus allows the column to be operated at convenient flow ratios.

In practice, the acetamino-acids have always been present in dilute solution, and since the relative volumes of chloroform and water phases are known to be scarcely affected by the addition of acetic acid up to a concentration of 5%, we conclude that the same holds good for acetamino-acids, and in practice have found this conclusion justified. This makes possible treatment of the equilibria of each ternary system on a simple two-coordinate diagram, and renders the usual triangular diagram with tie-lines unnecessary. The coordinates employed have been concentration of solute (mg. per ml.) in the chloroform and water phases respectively.

The equilibria concerned have not been investigated experimentally in great detail, although there has been some addition to and revision of the data given by Synge [1939, 1]. This is appended to the present section.

It is clear that, at a given temperature, two distinct factors will cause the equilibrium curve for a carboxylic acid in chloroform-water to depart from the straight line obtaining under Henry's law. These are (1) ionic dissociation of the solute in the aqueous phase, and (2) association of the solute to double molecules in the chloroform phase.

Smith & White [1929] have investigated the distribution of various carboxylic acids between organic solvents (including chloroform) and water, and have shown that the experimentally obtained curves agree very closely with those calculated by applying the mass action law to the dissociation in the aqueous phase and association in the organic solvent phase, on the assumption that unionized single molecules are distributed between the phases according to Henry's law.

In the present investigation we have studied the distribution of acetyl-*dl*-leucine between chloroform and water phases at 37°.

Assuming an ionization constant of $2 \cdot 3 \times 10^{-4}$ (as recorded for acetylglycine in Landolt-Börnstein's 'Tabellen') it is possible to calculate the concentration of unionized acetylleucine in the aqueous phase from its total concentration, according to Ostwald's dilution law. It will be seen from Table 1 that the ratio

$$D = \frac{\text{Concentration of unionized solute in aqueous phase}}{\text{Concentration of solute in chloroform phase}},$$

remains, within the experimental error, constant over the range studied. No accurate value can therefore be given from these data for the association constant for acetylleucine in chloroform.

Table 1. *Distribution of acetyl-*dl*-leucine between chloroform and water at 37°*

Acetylleucine in water phase millimol./ml.	Acetylleucine in $CHCl_3$ phase millimol./ml.	Percentage ionization	Unionized acetylleucine in water phase millimol./ml.	D
0·0442	0·00462	7·2	0·0410	8·9
0·0242	0·00227	10·0	0·0218	9·6
0·0200	0·00210	10·7	0·0179	8·5
0·0118	0·00104	14·0	0·0102	9·8
0·0055	0·00048	20·5	0·0044	9·2

From this it is clear that in dilute chloroform solution association of acetylleucine to double molecules is insignificant. This is not necessarily the case for other acetamino-acids, although Smith & White have shown (Table 2) that for the series of fatty acids in chloroform the association equilibrium constant is of the same order throughout. In this table

$$K = \frac{(\text{Conc. single molecules})^2}{\text{Conc. double molecules}} \text{ as mg.mol. per ml.}$$

Table 2. *Association of fatty acids in chloroform at 25°*

Acid	K	Acid	K
Propionic	15·3	—	—
Butyric	10·1	*iso*Butyric	9·1
Valeric	10·1	*iso*Valeric	7·6
Hexoic	17·0	*iso*Hexoic	9·2

It is interesting to note that Smith & White found K for α-bromopropionic acid to be 3900, i.e. that association to double molecules is much inhibited by the presence of the α-bromo group. It is possible that the α-acetamino group has the same effect.

We have compared the partition coefficients of optically active and racemic acetamino-acids in one case only (Table 3). Here no difference was observed.

Table 3. *Distribution of acetyl-*d(+)*-leucine between chloroform and water at 37 (cf. Table 1)*

Acetylleucine in water phase millimol./ml.	Acetylleucine in $CHCl_3$ phase millimol./ml.	Percentage ionization	Unionized acetylleucine in water phase millimol./ml.	D
0·0132	0·00125	13·8	0·0114	9·1
0·0069	0·00068	18 5	0·0056	8·3

We considered at one stage the possibility of using acetic acid as a solvent for acetamino-acid mixtures during injection into the chloroform-water extraction train. A preliminary experiment on the effect of acetic acid on the distribution of acetylleucine between chloroform and water phases showed that it caused the acetylleucine to enter the chloroform more readily. The results of this experiment are summarized in Table 4.

Table 4. *Effect of acetic acid on the distribution of acetyl-*dl*-leucine between chloroform and water phases at 37°*

The system contained 10 ml. water, 30 ml. chloroform and 56 2 mg. acetyl-*dl*-leucine, to which was added before equilibration the amount of acetic acid shown in the first column of the table.

Acetic acid added mg.	Acetylleucine in water phase mg. ml.	Acetylleucine in chloroform phase mg./ml.	P
0	4 18	0·393	10·6
50	4·20	0·497	8·45
150	3·98	0·500	7·96
400	3·58	0·700	5·11

This effect is presumably due to the formation of the double molecule ($CH_3.COOH.(CH_3)_2.CH.CH_2.CHNHAc.COOH$) in the chloroform phase.

The presence of considerable amounts of acetic acid in the extraction of acetylated protein hydrolysates and amino-acid mixtures by chloroform in the Neuberger [1938, 1] apparatus [cf. Synge, 1939, 2] is thus seen to be of assistance to the extraction, which is also aided by the large quantities of salt present in the aqueous phase.

In applying these equilibrium data to mixtures of acetamino-acids, the following points must be made:

(1) The degree of ionization in the aqueous phase will depend on the total concentration of acetamino-acid present in that phase. If the ionization constants of all the acetamino-acids present are the same, the degree of ionization of each component of the mixture will be the same, and will depend on the total molar concentration of the components. Thus the ratio of the partition coefficients will be unaffected by dilution, although the absolute value will change.

(2) Association to double molecules in the chloroform phase will, where it occurs, render the substances more readily extractable into chloroform. In mixtures, 'mixed double molecules' may be expected, and the equilibrium

constant for their formation is not predictable. Since the effect seems likely to be small compared with the ionization effect under the conditions used by us, we have not subjected this question to experiment.

In the light of these equilibrium data, it is possible to calculate what degree of separation may be expected when a mixture of two acetamino-acids is fractionated in a multi-plate chloroform-water extraction train. In designing the column described in the present paper we employed the McCabe-Thiele diagram for estimating the number of plates necessary to give the required separation. The following example will illustrate its use.

A column of forty theoretical plates with central feed of an undiluted 1 : 1 mixture of acetyl-*dl*-leucine and acetyl-*l*-proline hydrate is assumed, as this has some bearing on our own experiments. The column is operated at 37°. At this temperature, *D* for acetyl-*dl*-leucine is 9·2, for acetyl-*l*-proline 17·2 (mean value of determinations). The equilibrium curves for these two substances are plotted separately (Figs. 1 and 2), assuming the ionization in the aqueous phase to follow Ostwald's dilution law, and to have the ionization constant $2{\cdot}3 \times 10^{-4}$ *M*.

The flow ratio employed is the geometric mean of the two values of *D*, i.e. $\frac{\text{Rate of flow of chloroform}}{\text{Rate of flow of water}} = 12{\cdot}58$, and is taken as constant throughout the column.

McCabe-Thiele diagrams are constructed for each solute separately (Figs. 1 and 2). The location of the operating lines is determined as follows:

(1) Both lines (rectifying and analysing) are parallel, and have a slope of 1/12·58.

(2) The operating lines cut the axes at the concentration of solute in the chloroform and water outflows respectively.

(3) Twenty theoretical plates may be drawn between each operating line and the equilibrium line from the point at which the operating line cuts the axis to the point on the equilibrium line corresponding to the feed-plate.

In Figs. 1 and 2 the separation of the two solutes is treated individually. In Fig. 3 the individual and total concentrations of the two solutes on each plate, as given by Figs. 1 and 2, are plotted.

It will be seen that the curvature of the equilibrium lines in very dilute solution causes the acetylproline to flow out almost completely in the water outflow, whereas only 97·6 % of the acetylleucine flows out in the chloroform, and the rest in the water.

Since, however, the degree of ionization will depend on the total concentration of acid in each plate, it will be seen from Fig. 3 that this does not fall below 0·02*M*, and the degree of ionization will therefore at no point of the column be greater than 10 %. The equilibrium lines will therefore approximate more closely to straight lines through the origin, and the separation will be more nearly that calculated on the assumption that Henry's law operates, i.e. 99·6 % of each solute will emerge from the column at one end and 0·4 % at the other.

Under conditions of batch operation, where the mixture to be fractionated is added in one lot initially at the middle of the column, the separation cannot be so readily predicted, although the work of Daniels & Martin suggests that it should not differ widely from that obtained under conditions of continuous operation.

In batch operation, dilution will cause any component of the mixture to move more readily towards the water outflow end of the column; complete removal will therefore be more readily obtainable for the components of the mixture flowing out largely in the water than for those flowing out largely in the chloroform. We therefore intend in future to operate the column in a manner

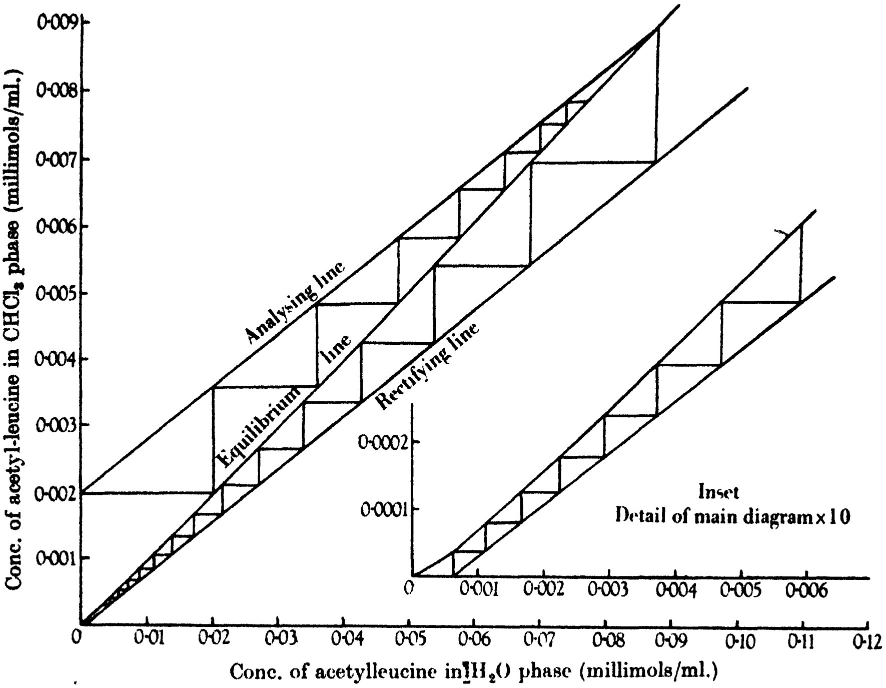


Fig. 1. McCabe-Thiele diagram showing distribution of acetylleucine.

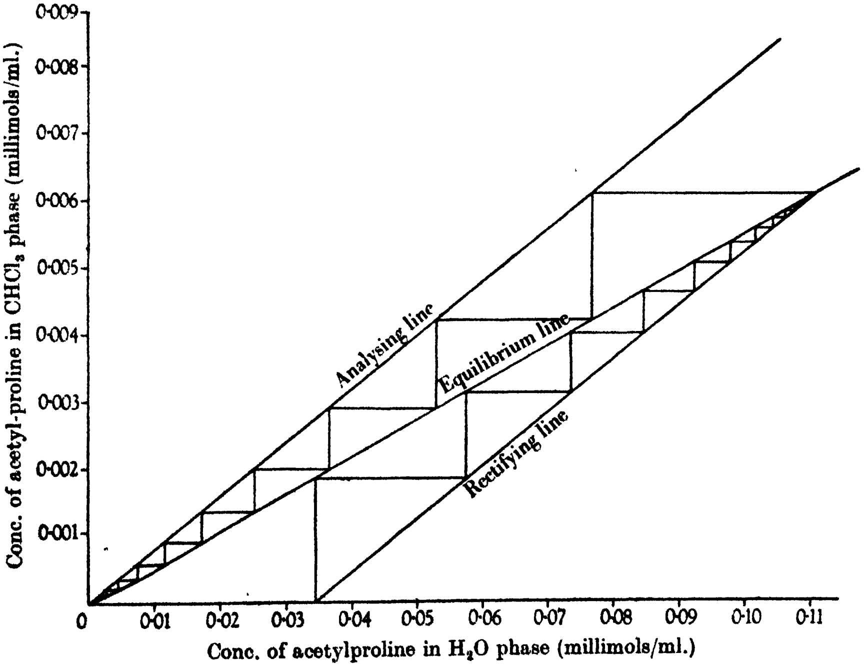


Fig. 2. McCabe-Thiele diagram showing distribution of acetylproline.

directly analogous to batch fractional distillation, i.e. by adding the mixture initially at the chloroform outflow end of the column, and maintaining its concentration there by evaporation of solvent without withdrawal of solution. Gradual increase of the rate of water flow through the column should then lead to sharp fractionation of the material obtained in the water outflow from the column. In addition, the effective length of the column would be twice that under the conditions of operation described in the present paper, since the column would be completely rectifying, whereas under the present conditions each half of the column serves as analyser for the rectifying action of the other half.

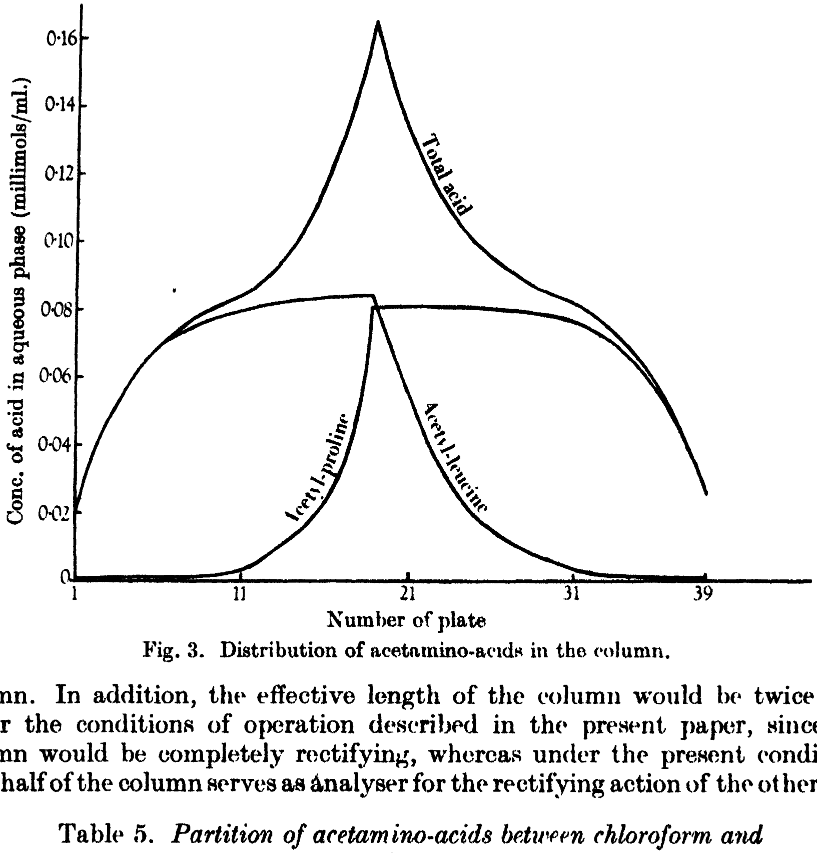


Fig. 3. Distribution of acetamino-acids in the column.

Table 5. *Partition of acetamino-acids between chloroform and water phases at 37°*

Compound	Conc. in water phase mg./ml.	Conc. in chloroform phase mg./ml.	*P*
Acetyl-*l*-phenylalanine	1·44	0·37	3·9
	0·76	0·18	4·2
Acetyl-*d*-*nor*leucine	1·74	0·27	6·45
	0·94	0·13	7·2
Acetyl-*d*-*iso*leucine	1·86	0·25	7·4
	0·98	0·11	9·0
Acetyl-*d*-leucine	2·28	0·22	10·5
	1·20	0·12	10·0
Acetyl-*l*-valine	3·3	0·22	15
Acetyl-*l*-proline (hydrate)	3·03	0·16	18·9
	1·55	0·09	17·2
Acetyl-*l*-methionine	3·04	0·11	27·6
	1·46	0·045	32·4
Acetyl-*dl*-α-aminobutyric acid	3·42	0·05	68
Acetyl-*dl*-alanine	4·74	0·022	216

New data on the partition of acetamino-acids between chloroform and water

In connexion with the present work it was desirable to redetermine and supplement some of the data for partition coefficients given by Synge [1939, 1]. The figures given in Table 5 were obtained by gravimetric determination of the solute in each phase after equilibration in a separating funnel.

The main discrepancy between the present figures and those previously given is in the figure for valine. The actual separations described below gave valine a place intermediate between proline and methionine. It is possible that the partition coefficient of its acetyl derivative alters more rapidly on dilution than that of proline.

Description of the machine

In the apparatus employed for the present work, the column in which water and chloroform phases flow counter-current consists of 40 units in series. Fig. 4 illustrates a single unit. The vertical or 'stirring' tubes (1, Figs. 4 *B*, 5, 6, 7) and the inclined or 'settling' tubes (2, Figs. 4 *B*, 5, 6, 7) connecting successive stirring tubes are made of pyrex glass. Each stirring tube holds a pair of silver stirrers (3, Fig. 4), each being a perforated dished disk of 0·017 in. silver sheet riveted to hard-drawn 16 s.w.g. silver wire. (Silver, unlike nickel and silver solder, is not attacked under the conditions of operation of the column.)

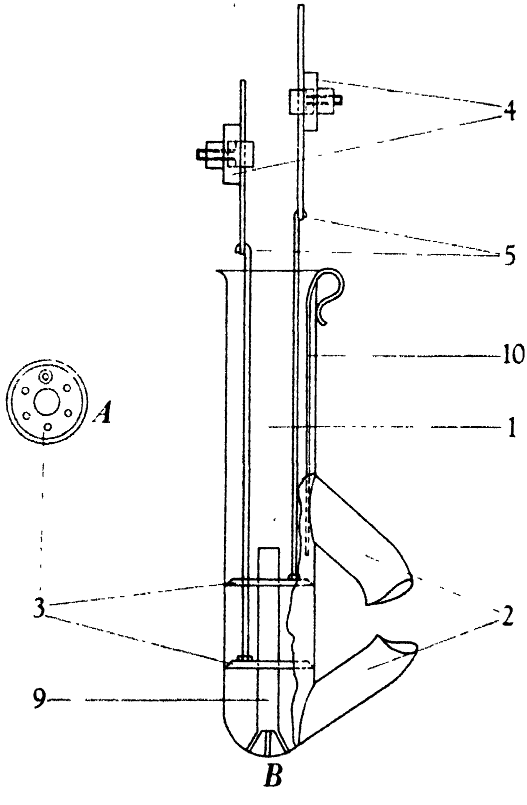


Fig. 4. Single unit of column.

Each stirrer is attached to a grid (4, Figs. 4 *B*, 5, 6, 7) through a universal joint (5, Figs. 4 *B*, 7). The two grids, and thus each pair of stirrers, are given equal and opposite reciprocating motions by a system of links (Watt's parallel motion) (6, Figs. 5, 6, 7). This is driven through a variable-throw crank (7, Fig. 8) and sprocket gear by a 1/4 H.P. induction motor (8, Fig. 8) mounted below the table.

Fig. 5.

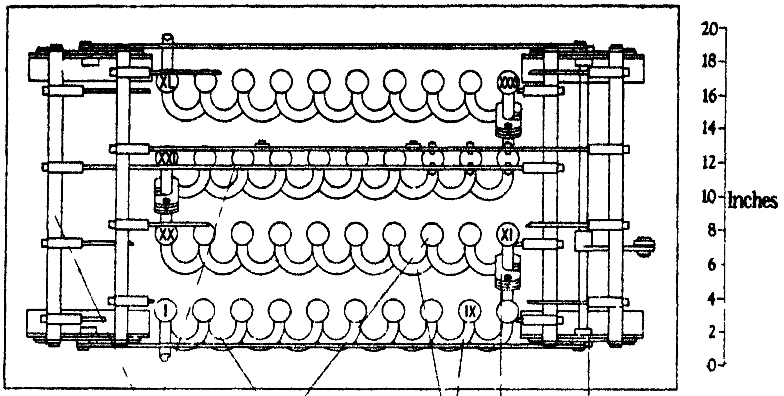


Fig. 6.

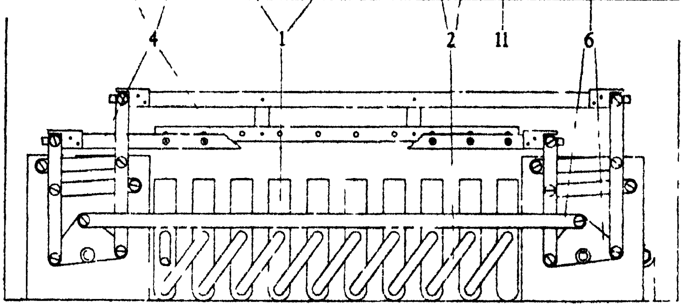


Fig. 5. Plan of column. Fig. 6. Side elevation of column.

A vertical glass tube (9, Fig. 4 *B*), open top and bottom and having three feet, rests on the bottom of each stirring tube and passes through the central perforation of each stirrer. A baffle plate (10, Fig. 4 *B*) of silver sheet is clipped to the rim of each stirring tube, and covers the mouth of the settling tube.

The stirring tubes are numbered 1–40 as shown in Fig. 5, and are sealed together in four banks of ten elements each. Successive banks are connected through a ground-glass flange joint, having a cellophane washer and held in position by an ebonite clamp (11, Figs. 5, 7). The chloroform and water entries (12 and 13) and exits (14 and 15) are arranged as shown in Figs. 7 and 8.

The column rests on a stoutly built table, and is held in position by light clips (not shown in drawing). The link-gear and the whole column (except for the water and chloroform exits) is enclosed in a wooden casing (16, Figs. 7, 8) resting on the table. The interior of this is maintained at 37° by thermostatically controlled electric lamps. The air is kept in circulation by an electric fan. The casing is provided with a lid (17, Fig. 8), windows (18, Fig. 8) and a small door near to tube 20.

On leaving the column, chloroform flows from the adjustable level outflow (14, Figs. 7, 8) through a tube provided with a tap for sampling (19, Fig. 8) to the chloroform recovery still, a 2 l. flask (20, Fig. 8) which is immersed in an electrically heated water bath (1 kW.). The vapour from the still passes through the cyclone trap (21, Fig. 8), which eliminates entrainment of drops of liquid and, after condensation in the reflux condenser (22, Fig. 8), the chloroform falls to the constant level vessel (23, Fig. 8). The reflux arrangement is adopted so that the chloroform shall enter the constant level vessel hot, and thus free from dissolved air. This prevents the formation of bubbles in the flowmeter.

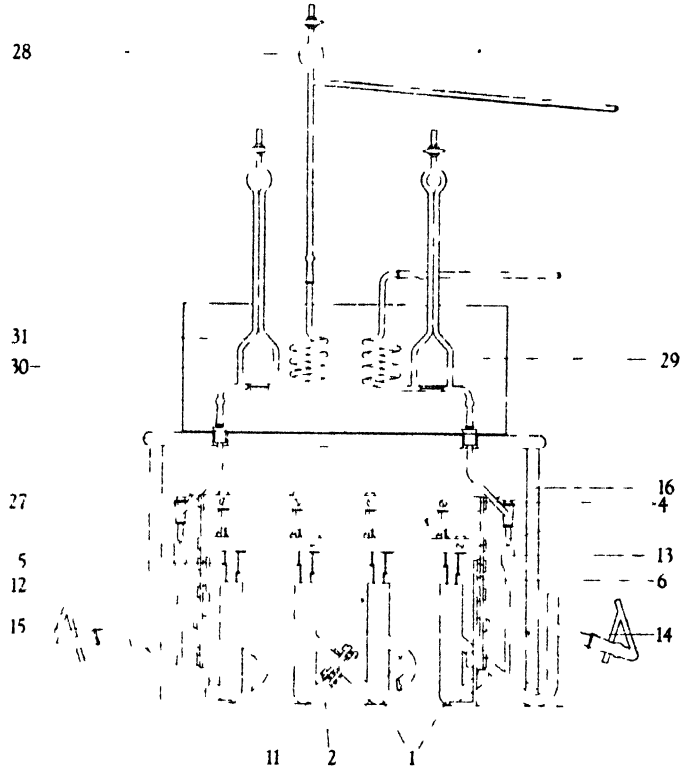


Fig. 7. End elevation of column.

The level of liquid in the constant level vessel is adjusted by raising or lowering the air inlet tube of the 10 l. Mariotte bottle (24, Fig. 8) which acts as the chloroform reservoir. The constant level vessel is connected to the reservoir and to the flowmeter through siphon tubes (25, Fig. 8); the siphons are maintained by differential pressure provided by a column of chloroform in the wash-bottle (26, Fig. 8) through which air is withdrawn from the Mariotte bottle. Chloroform is admitted to the column through the tap (27, Fig. 7). The rate of admission of chloroform is controlled by adjustment of the tap and of the chloroform level in the constant level vessel. Any water separating from the chloroform on its way to the column as a result of cooling is removed in the trap (28, Figs. 7, 8) below which the chloroform supply tube enters the water bath (29, Figs. 7, 8), which is thermostatically maintained at 37°. The chloroform passes through the heating coil (30, Fig. 7) and the capillary flowmeter (31, Figs. 7, 8) before passing to the column.

The same arrangement (not shown in drawing) is used for supplying water to the column; here de-aeration is effected by an electric immersion heater (60 W.) placed in the constant level vessel.

The gear for driving the stirrers was constructed for us by Unicam Instruments, Ltd., Cambridge, and the glass part of the column was made by Messrs A. Gallenkamp, London. We are grateful to their glass-blower, Mr J. Holme, for the efficient execution of this work. We wish also to thank Messrs H. Hall and E. Benton, of the Biochemical Laboratory, Cambridge, and Mr S. Dilworth of Torridon for their help in building the apparatus.

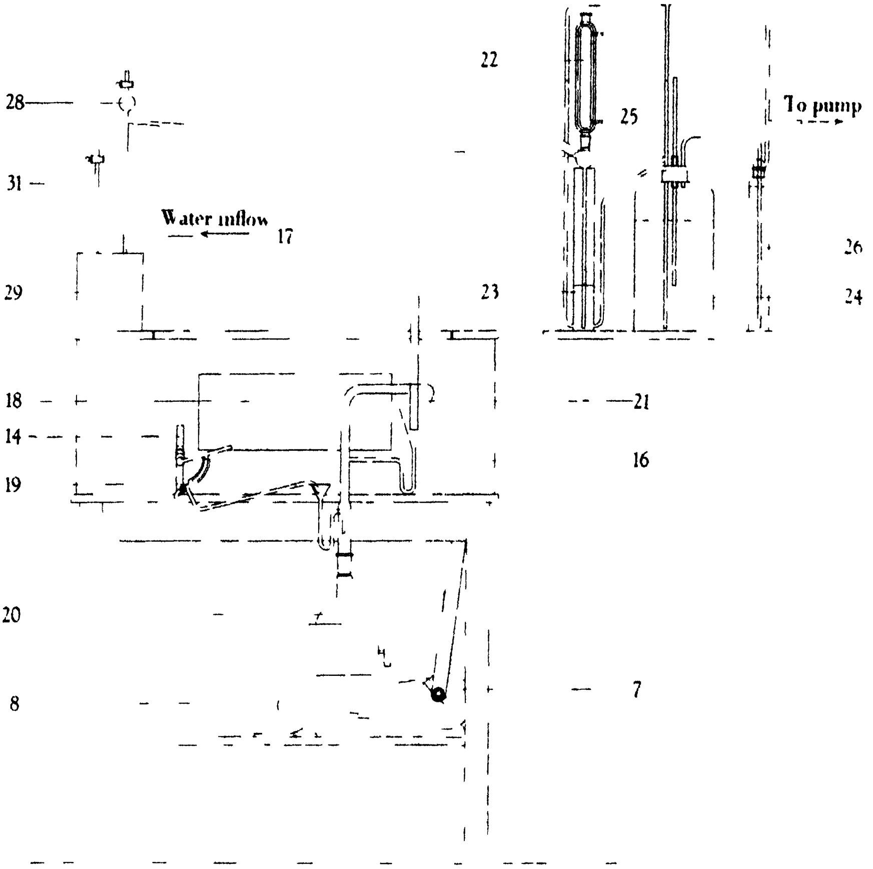


Fig. 8. General view of apparatus.

Operation of the column

When the column is in action, the motion of the stirrers maintains in each stirring tube an emulsion of which the aqueous is the disperse phase. Drops of water thus rise through the settling tube which leads to the next stirring tube. In this way a continuous stream of water passes along the column from tube 1 to tube 40. At the same time, chloroform flows in the opposite direction, under constant conditions the flow of each solvent will be uniform along the whole length of the train, and will depend on the rate at which the solvents are admitted to the column. The solvents leave the column by the adjustable-level outflows.

It will be appreciated that in addition to their normal function of contacting the liquids, the stirrers serve to pump the liquids along the column by continually displacing, in the stirring tubes, the chloroform and water phases from the positions that they would occupy at rest, thus providing gravitational potential energy for the settling process. This feature of design makes it unnecessary for the column to be tilted [cf. Cornish *et al.* 1934] and thus makes possible the present compact arrangement.

Although the stirrers in each tube are always moving equally in opposite directions, they tend to have, in addition to their stirring action, a 'dynamic' pumping action, which depends on:

(1) The orientation of the dished rim of the stirrers, as there is greater resistance to the motion of the stirrer in one direction than in the other.

(2) The asymmetry of motion of the link gear: the motion is not simple harmonic, and the stirrers approach each other at a different speed from that at which they separate. This effect can be reversed by attaching each stirrer to the other bar.

(3) The length of stroke and velocity of the stirrers, being greater for long strokes and high velocities.

(4) Possible differences in the viscosity of the emulsion at the different levels in the tube at which each stirrer is situated.

This pumping effect, being additive from tube to tube, can, if present in the same sense in a succession of tubes, so alter the level of liquid at different points in the column as to render it inoperative. It is greatly reduced by the central glass tubes, which prevent the establishment of serious pressure differences across any pair of stirrers, and are included for this reason as well as for convenience in emptying the column.

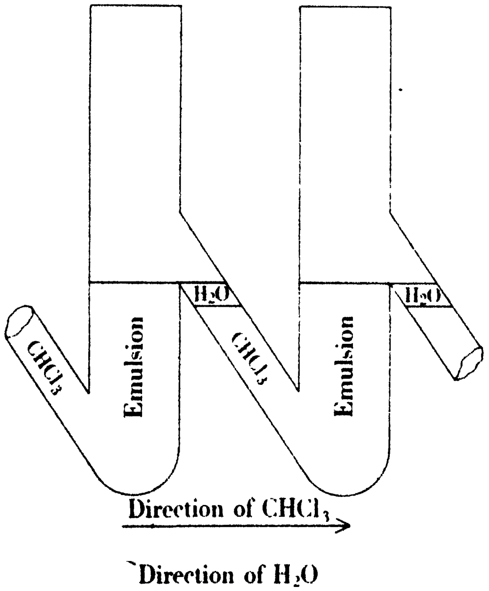


Fig. 9. Normal running of column.

If, as is the case when water is the disperse phase, the density of the emulsion in a stirring tube is less than that of the emulsion in the settling tube, there will be a tendency to 'static' pumping of liquid along the column towards tube 1. The level of the top of the emulsion in each stirring tube will thus tend to its low limit, i.e. the level of the lip of the settling tube leading out of it, as shown in Fig. 9. In practice, at low flow rates of chloroform, the surface can fall lower than this by splashing of chloroform over the lip into the settling tube. In this way the surface level of the liquid may fall to the level of the top stirrer, and splashing then becomes so bad that liquid is actually lost from the column. This is prevented by placing a silver baffle plate in front of the mouth of each settling tube, which checks the passage of liquid by splashing, and at the same time serves to stop 'entrainment' of chloroform by back-mixing between successive units. Thus, at low flow rates of chloroform, each settling tube will have a layer of water at its top and each stirring tube will be full to the lip of the settling tube (Fig. 9), so that the average density of liquid in each limb is equal.

Under these conditions, the level of liquid in each stirring tube will be at its low limit, and this is the most desirable condition in which to operate the column. Stirring is less efficient when the level of the liquid in the stirring tubes rises, as is bound to happen on increasing the rate of chloroform flow, owing to the head required for overcoming the resistance of the column to flow. This rate can, however, be increased without impairing the operation of the column until it cancels out the effect of the 'static pumping', i.e. until there is no separated layer of water at the top of the settling tubes.

The rate of passage of water out of each stirring tube, and thus along the column, is normally determined by the bottom aperture of the corresponding settling tube, by the opposed rate of flow of chloroform (increase of which decreases the speed of settling), and by the concentration and state of division of the water phase at the bottom of the stirring tube. These depend on the efficiency of the stirring and on the surface tension of the emulsion, which may be lowered by the presence of dissolved substances. If, as occasionally happens, the presence of these causes excessive emulsification, the rate of flow of solvents may be determined entirely by the rate of settling in the settling tubes.

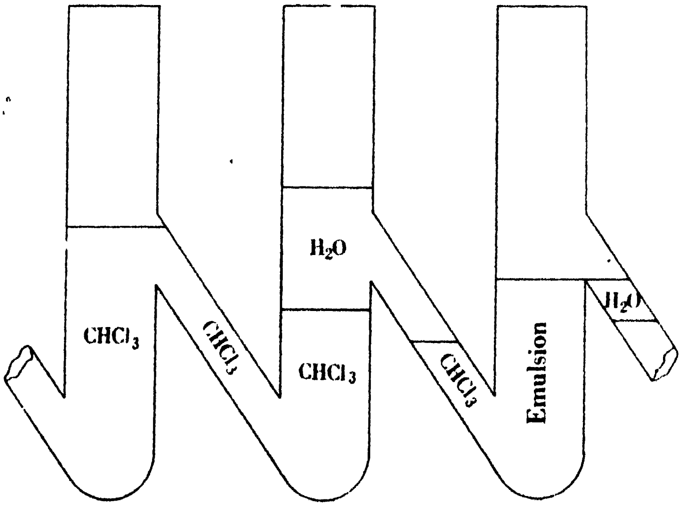


Fig. 10. Water blockage of column.

In order to get efficient stirring throughout the tube, the vertical position of the stirrers must be carefully adjusted. This is particularly necessary when the stirrers are operating on a short stroke (at correspondingly greater speed); these are the most favourable conditions for the elimination of 'dynamic' pumping and splashing.

When more water enters a stirring tube than it is capable of passing, water collects at the top of the tube, raising the level in the tube and preventing the flow of chloroform. This causes accumulation of chloroform between the defective unit and the chloroform inflow, as shown in Fig. 10. This type of blockage is more difficult to deal with than a block due to excessive flow of chloroform, as the column responds more slowly to changes in the rate of water inflow than to changes in the rate of chloroform inflow. Moreover, in the course of eliminating a 'water block' (and sometimes in ordinary operation, when water-flow is being established through the column) the column may exhibit a cyclical instability, since the removal of the 'water block' may be followed by a period of intense 'static pumping', followed by the re-establishment of the block farther along the column. These phenomena involve fluctuations in the flow ratio at different points of the column, and are avoided as far as possible by working with a low level of liquid in the column and moderate rates of solvent-flow.

Working and testing the machine

In working the machine, we proceed as follows. The water and air thermostats being at 37°, chloroform is admitted until the column is full. The rate of admission of chloroform to the column is then adjusted, and the stirrers are set in motion (stroke 0·75 cm. at 390 vibrations per min.). The required flow of water is then started. After running in this manner for some hours, constant conditions will be established throughout the column. Since, however, the addition of the material to be analysed lowers the surface tension between the phases, thereby increasing emulsification and the rate of flow of water, the material is usually added to the column at tube 20 as soon as the advancing stream of water has reached tube 27–29. This immediately liberates water from the middle part of the column, which rapidly finds its way towards tube 40, and an approximately steady state is thus established before water begins to leave the column.

At the end of the run, the material that has emerged in the chloroform is collected from the flask of the chloroform recovery still, while that which has emerged in the water is obtained by concentration *in vacuo*. The fraction remaining in the column is obtained by emptying the column and concentrating separately the two phases so obtained.

Although Cherbuliez & Wahl [1925] have found benzamino-acids to be volatile in steam, we have examined for N the distillates obtained in the concentration of solutions of acetamino-acids with negative results.

The solvent volumes in the column are nearly independent of the flow ratio employed. 3·0–3·5 l. of chloroform and 150–350 ml. of water are the usual amounts.

Satisfactory rates of solvent flow for the range of flow ratios employed are shown in Table 6.

Table 6. *Solvent rates for different flow ratios in the column*

Flow ratio	Chloroform rate ml./min.	Water rate ml./min.
68	22·5	0·33
20·8	18·75	0·86
13·9	19	1·37
8·0	8·0	1·00

Since on the average each molecule of chloroform or water will spend about 3 min. in each unit of the column, and as intimacy of mixing in the stirring tubes is greater than in the ordinary laboratory separating funnel, it seems likely that each unit will correspond nearly to one theoretical plate, assuming no entrainment to occur. By the use of red ink, the pigment of which stays completely in the water phase, we have never detected any entrainment of water, while the use of Sudan III, which stays completely in the chloroform, has shown that entrainment of chloroform is only detectable at the lowest rate of chloroform flow employed; under these circumstances it is not great, and only occurs in the absence of the baffle. The main factor vitiating the performance of the machine would seem to be local variations in the flow ratio, particularly those occurring at the beginning of the run.

Although precautions were taken against loss of chloroform by evaporation by sealing cracks, etc. in the walls of the air-thermostat box with plasticine, by packing wool around the entry of the driving-arm to the box, and by providing a rubber seating for the box lid, they were not completely successful, the total loss of chloroform on a 24 hr. run amounting to about 1·5 l.; this is not surprising when it is remembered that the equilibrium weight of chloroform vapour in the box at 37° is about 500 g. This loss corresponds to about 1 ml. per min. of chloroform, and most of it must be assumed to take place along the length of the column. Its effect on the operation of the column is greatest at low flow ratios, and is to produce a continuous change of flow ratio along the length of the column, amounting, at flow ratio 8, to a change of 12·5 %.

In operating the machine in most of this work we placed the material to be analysed in the middle of the column in solid form in one batch. Larger quantities could be added in a continuous manner; the machine is just as capable of continuous as of batch operation. For this purpose tube 20 is connected through a capillary to an electrically driven glass syringe. The problem is to find a suitable solvent for the acetamino-acids which will permit the slow injection of a concentrated solution. The mixtures obtained from proteins crystallize from concentrated aqueous solution, and glacial acetic acid, though a good solvent, distils with the chloroform, and thus accumulates in the column, seriously affecting the equilibria, as already pointed out. This effect is strikingly demonstrated by comparing the results of tests 1 and 2 given below.

Test 1 is also of interest because the time course of emergence of the substances to be separated was studied. In other experiments we have only collected the three fractions at the end of the run.

Possibly alcohol would prove a more desirable solvent for injection.

Tests. For testing the machine, a 1 : 1 mixture of acetyl-*l*-proline hydrate [du Vigneaud & Meyer, 1932, 1, 2] and acetyl-*dl*-leucine [Fischer, 1901] was employed. These substances have the partition coefficients given in Tables 1 and 5, which are sufficiently close to give a good test of the machine, and in addition small amounts of each in the presence of the other can be conveniently detected, the former by its large negative rotation, and the latter by determination of amino-N after acid hydrolysis.

Test 1. The column was allowed to reach a steady state at flow ratio 13·9 (solvent rates as in Table 6). A solution of 4·5 g. of acetyl-*l*-proline hydrate and 4·5 g. of acetyl-*dl*-leucine in 25 g. of glacial acetic acid was then injected at a steady rate, 12 hr. being required for complete injection. The chloroform and water effluents were collected at the intervals shown in Table 7, which shows cumulatively the amount of each solute (as % of amount finally injected) which had emerged at different times.

Table 7

Time from commencement of injection hr.	In chloroform effluent		In water effluent	
	% of acetyl-leucine	% of acetyl-proline	% of acetyl-leucine	% of acetyl-proline
4	0·46	0·0	0·00	1·0
6	2·7	0·1	0·02	1·8
8	14·0	0·6	0·07	3·2
10	40·4	3·4	0·13	5·1
12	65·9	9·3	0·24	6·7
15	92·4	25·2	0·38	8·7
19	98·8	41·2	0·50	11·5

Test 2. Column as in test 1. A mixture of 1·04 g. acetyl-*dl*-leucine and 1·02 g. acetyl-*l*-proline hydrate was added in the solid form, and the machine was allowed to run for 11 hr. At the end of this time there had emerged in the chloroform 26·5 % of the acetylleucine and 0·60 % of the acetylproline. In the same time there had emerged in the water 4·36 % of the acetylleucine and 48·7 % of the acetylproline.

The wide divergence between tests 1 and 2 may be ascribed to the presence of acetic acid in test 1. The accumulation of this has the effect of rendering both solutes more readily extractable by chloroform.

These tests gave a rough idea of what degree of separation was to be expected from the machine, and we were able on this basis to use it for the separation of more complex mixtures.

Separation of a known mixture of amino-acids

The possibility of using liquid-liquid extraction for separating and estimating some of the monoamino-acids occurring in protein hydrolysates was briefly discussed by Synge [1939, 2], who described a procedure for obtaining a good yield of the required amino-acids in the form of their acetyl derivatives and free from salt. Such a mixture is suitable for fractionation in the machine which we describe in this paper.

Before attempting to use the new technique for the analysis of a protein it was considered advisable to control it by experiment with a known mixture of amino-acids.

The preliminary fractionation of such a mixture was described by Synge [1939, 2], who stated: 'The mixture was intended to simulate a gelatin hydrolysate, and lacked cystine and tryptophan. It would obviously be desirable to repeat the experiment with a mixture containing these amino-acids, as they are responsible for much secondary change when such a mixture is boiled in acid solution.'

The present investigation has been conducted with special reference to wool, in which both these amino-acids are present, cystine being a major constituent. A mixture was made up containing all the amino-acids recorded in the literature as constituents of wool, with the exception of methionine and phenylalanine. The former was omitted because its behaviour had already been studied [Synge, 1939, 2] and its absence permitted a study of the behaviour of cystine during the fractionation by means of total S determinations.

The amino-acids were in roughly the proportions recorded for wool.

All samples used for analysis were aliquots; allowance has been made for the samples removed at each stage for analysis, and all figures given are on the basis of the original mixture, as detailed in Table 8.

All evaporations were carried out *in vacuo* below 40°.

The values given for 'amino-N' refer to amino-N determined by the Van Slyke procedure on samples hydrolysed with 2N HCl for 3 hr. at 100°, and subsequently neutralized with excess of sodium acetate. Where the material had been dissolved in alcohol, great care was taken to remove this completely before determining amino-N.

Table 8. *The mixture of amino-acids used in the experiment*

Compound	N (g.)	N as % of total N
Glycine	0·290	12·8
dl-Alanine	0·137	6·0
dl-Valine	0·091	4·0
l-Leucine	0·206	9·0
dl-Serine	0·070	3·1
l-Proline	0·128	5·6
l-Aspartic acid	0·053	2·3
l-Glutamic acid	0·190	8·3
l-Histidine (as hydrochloride)	0·048	2·1
l-Arginine (as hydrochloride)	0·482	21·2
dl-Lysine (as dihydrochloride)	0·109	4·8
l-Tyrosine	0·083	3·6
l-Cystine	0·242	10·7
l-Tryptophan	0·026	1·1
Ammonium chloride	0·124	5·4
Total	2·279	100

The mixture shown in Table 8 was dissolved in 200 ml. 6*N* HCl, and was refluxed for 24 hr. The resulting solution was concentrated and acetylated exactly as described by Synge [1939, 2], using 400 ml. 2*N* NaOH and 40 ml. of acetic anhydride. After keeping overnight, the mixture was acidified as described, transferred to the Neuberger extractor, and extracted with approximately 30 vol. of chloroform. The aqueous residue was freed from salt and re-acetylated exactly as described, acidified, and again extracted with 30 vol. of chloroform in the Neuberger extractor. The two chloroform extracts were combined and evaporated to dryness; the residue was made up to standard volume in water (fraction E).

This fraction contained 24·7 % of the total N of the mixture.

It was completely free from $SO_4^=$, and contained 42 mg. S (7·6 % of the cystine-S of the mixture). We are grateful to Mr W. R. Cuthbertson for this S determination.

Fraction E was evaporated to dryness in a vacuum desiccator, and the resulting toffee-like solid was fractionated in the machine for 24 hr. at flow-ratio 68, the geometric mean of the partition coefficients of acetylalanine and acetylproline. In this and subsequent fractionations the solvent rates employed were those given in Table 6.

Table 9 shows the distribution of N, S and amino-N in the three resulting fractions.

Table 9

Fraction	N as % of total N	S as % of original cystine S	Amino-N as % of total N
68_c (in chloroform)	17·9	2·2	12·7
68_m (in column)	0·8	—	—
68_w (in water)	5·9	5·0	—

Fraction 68_w was evaporated to dryness. It was obviously a complicated mixture, and crystallized very slowly. By successive crystallization from ethyl acetate and acetone a small amount of acetylglycine was isolated. The fraction might be expected to contain also acetylalanine, *N*-acetyltyrosine, diacetylcystine, diacetyllysine and possibly other constituents. It was not further investigated.

Fraction 68_m was not further investigated.

Fraction 68_c was evaporated to dryness, to give a practically completely crystalline residue, coloured pale yellow. It was fractionated in the machine for 24 hr. at flow-ratio 13, giving the fractions shown in Table 10.

Table 10

Fraction	N as % of total N	Amino-N as % of total N
68_c13_c	2·7	—
68_c13_m	3·2	—
68_c13_w	10·2	5·8

68_c13_m *and* 68_c13_c both obviously consisted almost completely of acetylleucine, and were therefore combined before working up.

The material consisted of crystals accompanied by a brown oil, which was later found always to be produced in the course of fractionation in the machine. Its nature is discussed in the succeeding section dealing with the analysis of wool. It could readily be separated from the crystals by washing them with chloroform or ligroin. By subsequent recrystallization from chloroform and from water, three crops were obtained:

Crop I. 1·25 g. M.P. 182°. $[\alpha]_D^{24^\circ} -23{\cdot}5^\circ$ (alcohol, $c=3{\cdot}3$). (Found: C, 56·2; H, 8·54; N, 8·23 %. Calc. for $C_8H_{15}O_3N$: C, 55·5; H, 8·67; N, 8·1 %.)

Crop II. 0·070 g. M.P. 155–160°. $[\alpha]_D^{24^\circ} -5{\cdot}2^\circ$ (alcohol, $c=1{\cdot}0$). (Found: C, 55·9, H, 8·53; N, 7·8 %.)

Crop III. 0·162 g. Not further investigated.

Synge [1939, 1] records acetyl-*d*-leucine as having M.P. 186–188°; $[\alpha]_D^{22^\circ} +23{\cdot}2^\circ$ (alcohol). Fischer [1901] records acetyl-*dl*-leucine as having M.P. 161°.

From this it seems that the crystalline part of this fraction consists of acetyl-*l*-leucine which has undergone about 15 % racemization.

89 % of the N of fractions 68_c13_m and 68_c13_c is thus accounted for as crystalline acetylleucine.

68_c13_w. On concentrating an aqueous solution of this fraction, the first crop of crystals (0·353 g.) had M.P. 180° and this was not depressed on admixture of acetyl-*l*-leucine from the other two fractions.

The next crop obtained melted at 130–137°, and the next at 110–140°. From these it proved possible, by repeated crystallization from water, to isolate 0·269 g. of a product having M.P. 147–148°. This was not depressed by admixture with authentic acetyl-*dl*-valine [Synge, 1939, 1]. (Found: C, 52·5; H, 8·22; N, 8·90 %. Calc. for $C_7H_{13}O_3N$: C, 52·8; H, 8·17; N, 8·8 %.)

Since it was clear that the mother liquors contained a mixture of acetylleucine, acetylvaline and acetylproline, and since acetylproline had already been satisfactorily isolated in the analysis of wool (see below), this fraction was not further investigated. Its proline content could be judged by the difference between total and amino-N, shown in Table 10.

We draw the following conclusions from this experiment with a known mixture of amino-acids:

(1) Fraction E, on fractionation in the machine at flow-ratio 68, yields in fraction 68_c the amino-acids leucine, proline and valine in about 95 % yield. It

will be noted that the ratio of amino-N to total N for fraction 68_c is 0·71. Calculated from the original mixture, $\frac{\text{Leucine-N} + \text{valine-N}}{\text{Leucine-N} + \text{valine-N} + \text{proline-N}} = 0{\cdot}704$. *Methionine, leucine isomers and phenylalanine may be expected to behave similarly.*

(2) Racemization to the extent of about 15 % is to be expected. This probably occurs during acetylation, although the conditions of acetylation are so chosen as to reduce it to a minimum [cf. du Vigneaud & Meyer, 1932, 1]. The possibility of racemization of the free amino-acids on prolonged boiling with acid cannot be ignored. With cystine, this is known to be considerable, and Arnow & Opsahl [1940] and Johnson [1940] report slight racemization of glutamic acid under these conditions.

(3) 2–3 % of the S of the cystine in the mixture is carried into fraction 68_c. This cannot be in the form of diacetylcystine [cf. Synge, 1939, 1]. The state of combination of this S is discussed in the following section on the analysis of wool under fraction 68_m.

(4) Further fractionation of 68_c at flow ratio 13 largely freed about 70 % of the leucine from valine and proline, the bulk of which remained in fraction 68_c13_w. It might be expected that repeated fractionation in the machine at different flow ratios would achieve better separation. This was attempted with some success in the course of the work on the monoamino-acid fraction of wool described below.

Investigation of the higher monoamino-acids of wool

The literature on these constituents of wool is meagre. Abderhalden & Voitinovici [1907] investigated a wool hydrolysate by the ester distillation procedure of Fischer and reported figures for valine, proline and 'leucine' which are of interest in connexion with the present investigation. No attempt was made to decide whether *iso*leucine was present with the leucine. Phenylalanine could not be detected in the fraction in which it was expected to occur. Mueller [1923] described the isolation of methionine in small quantity from wool, and Barritt [1934] employed the volatile iodide method of Baernstein [1932] for its determination. Block [1939] reported a colorimetric determination of phenylalanine in wool. These analyses are compared at the end of this section with the results of the present investigation.

Commercial Merino 64*s* dry-combed top was extracted in a Soxhlet apparatus with benzene and alcohol successively. It was then washed several times with distilled water.

An amount containing 3·310 g. of N (approx. 20 g. of wool) was refluxed on an air bath with 200 ml. of 6*N* HCl for 25 hr. [cf. Marston, 1928].

In describing the subsequent analysis, allowance has been made for the aliquots removed at each stage. Wherever possible, amounts are expressed in N as % of total N of the original wool. Where absolute quantities are mentioned, they are to be referred to the original wool employed, containing 3·310 g. of N. In the later stages of the analysis the amounts of material actually employed corresponded to about 10 g. of wool.

Total N determinations on the various fractions were carried out by the Kjeldahl technique. N determinations on solid material were by the Dumas technique, and were carried out by Dr G. Weiler, Oxford, who is also responsible for the C, H and S determinations.

In the course of the acid hydrolysis a small amount of partly crystalline water-insoluble material was observed to collect in the condenser. It could be recrystallized from ether, in which it was very soluble. Suspecting the presence of a volatile organic acid, we diluted the hydrolysate when cool to 500 ml. and extracted it with 5 vol. of chloroform in the Neuberger extractor. The material which had collected in the condenser was combined with the hydrolysate before extraction.

An aliquot of the chloroform extract (which was free from Cl^-) was shaken with water, and the mixture was titrated to phenol red with NaOH. The alkali required was equivalent to 0·204 milliequiv. of acid in the whole extract.

Another aliquot of the extract was evaporated at 37° in the open in a tared dish. The material not volatile under these conditions corresponded to 83 mg. in the whole extract.

The rest of the extract was evaporated at atmospheric pressure; the non-volatile residue from this was suspended in water and subjected to steam distillation until 500 ml. of distillate had been collected. The acid not distillable under these conditions amounted to 0·08 milliequiv.

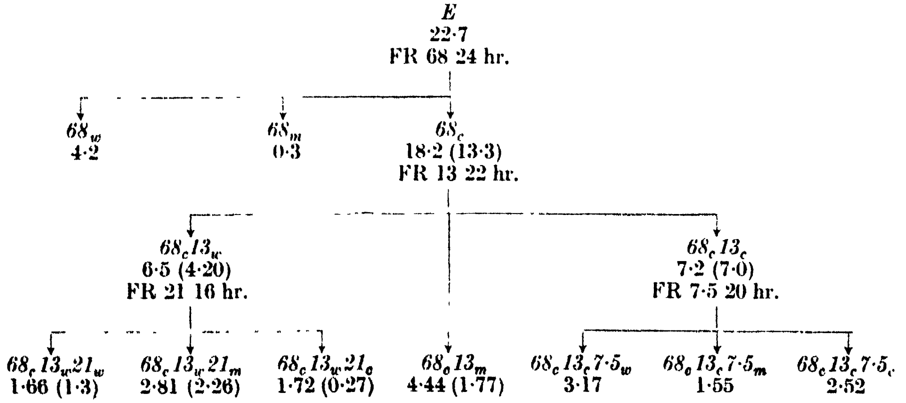
Steam distillation of the extract after neutralization to *p*H 7 with NaOH gave a small amount of water-insoluble material in the distillate.

In view of the insignificant quantities concerned, these fractions were not further investigated. We have indications that the amount of material extractable by chloroform from an acid hydrolysate of wool is increased by increasing the time and temperature of hydrolysis. Most of the material in question is therefore probably a product of secondary change.

After this extraction by chloroform, the hydrolysate was concentrated to dryness *in vacuo*. Two successive acetylations and extractions were carried out exactly as described above for the known mixture, and the combined extracts constituted fraction E, containing 22·7 % of the total N and 54·6 mg. of S (about 8 % of the total S of the wool).

Fraction E was then fractionated and sub-fractionated in the machine according to the scheme shown in Table 11. Between fractionations it was found convenient to handle the fractions by solution in alcohol. The nomenclature of the fractions is that employed in the previous section. The figures given immediately after each fraction in the table show its N content as % of total N, and the figures in brackets its amino-N content on the same basis, determined as in the previous section.

Table 11. *Sub-fractionation of fraction E*



The subsequent fractionation to which a fraction was subjected is shown in the table thus: *FR 68 24 hr.*

This means that the material in question was fractionated in the machine at flow ratio 68 (with solvent rates as given in Table 6) for 24 hr., after which the *three resulting fractions were collected.*

Non-amino-N of the fractions. This, the difference between total and amino-N, sets an upper limit to the proline present. It is calculated for the relevant fractions as shown in Table 12. In view of the good yields of crystalline acetylproline obtained in the investigation of the end-fractions, there is no reason for postulating the presence of any non-amino-N-containing substance other than proline.

Table 12. *Non-amino-N of the fractions*

Fraction	Non-amino-N as % of total N of wool
$68_c13_w21_w$	0·36
$68_c13_w21_m$	0·55
$68_c13_w21_c$	1·45
68_c13_m	2·67
68_c13_c	0·20
Total	5·23
Cf. 68_c	4·9

Methionine determinations on the fractions. These were kindly carried out for us by Mr G. Lindley, by the 'volatile iodide' method of Baernstein [1932; 1936]. The results are given in Table 13.

Table 13. *Methionine of the fractions*

Fraction	Methionine-N as % of total N of wool
68_c	0·52
$68_c13_w21_w$	0·31
$68_c13_w21_m$	0·09
$68_c13_w21_c$	0·01

Great care was taken to remove alcohol completely from the samples before subjecting them to analysis, since this would appear as volatile iodide in the course of the determination. There remained the possibility that handling the fractions with alcohol had induced partial esterification.

The distribution of volatile iodide in the end-fractions, however, makes it clear that acetamino-acid ethyl esters are not involved, since these are readily extractable from water by chloroform. It is however possible that 25 % of the volatile iodide determined on fraction 68_c may be due to this cause.

The final distribution of volatile iodide in the end-fractions is roughly what would be expected from a knowledge of the partition coefficient of acetylmethionine (cf. Table 5).

Detailed investigation of the end-fractions.

68_w. This did not readily crystallize, and being obviously a complex mixture was not further investigated.

68_m. No acetamino-acid could be crystallized from this fraction. It is in this fraction that α-aminobutyric acid [cf. Vickery & Schmidt, 1931] would be expected to occur, if present (cf. Table 5). We conclude from the low N content of this fraction that α-aminobutyric acid is absent from, or at most a very minor constituent of, wool.

In this and all other 'M' fractions we obtained by crystallization from alcohol, followed sometimes by recrystallization from ether, small quantities (never more than 20 mg. from a fraction) of a substance having M.P. 120–121°. (Found: C, 35·2; H, 5·65; S, 59·8 %; N absent. $C_3H_6S_2$ requires C, 34·0; H, 5·67; S, 60·4 %.)

This substance was insoluble in water and extremely soluble in chloroform and ligroin. It was accompanied by a brown oil, which remained insoluble in water on boiling with mineral acid. The oil from fraction $68_c 13_c 7{\cdot}5_m$ after boiling with 2*N* HCl had S, 5·9 %.

A compound of the same composition and M.P., to which the formula $C_6H_{12}S_4$ was ascribed, was reported by Tucker & Reid [1932] to result in low yield from the interaction of CH_2Cl_2 with $CH_2SH.CH_2SH$ (formed by the interaction of H_2S with $CH_2Cl.CH_2Cl$). They postulated a cyclic polymethylene sulphide structure.

Although it is hard to see how the same compound could have resulted under the conditions employed by us, it seems reasonable to suppose that the present sulphur-rich bodies may be formed by the interaction of H_2S, etc. (resulting from the breakdown of cystine) with halogenated hydrocarbons. We are informed by Dr K. Bailey that 3–4 % of the S of wool is to be found in an acid hydrolysate as elementary S.

It is difficult to see why substances so soluble in chloroform and insoluble in water should remain in the column of the machine, rather than leave it in the chloroform effluent. Perhaps they are strongly adsorbed by the chloroform-water interface, the area of which is considerable while the machine is in operation.

$68_c 13_w 21_w$. In view of the high estimated methionine content of this fraction (19 % of N of fraction) it was subjected to immediate acid hydrolysis, with a view to carrying out the $HgCl_2$ precipitation described by Pirie [1932]. Possibly because of the small total quantity of material, this procedure did not give a satisfactory precipitation. The mixture was accordingly treated with H_2S to remove Hg, Ag_2O to remove Cl^- and H_2S to remove Ag. The filtrate from Ag_2S was evaporated to dryness. The crystalline residue was refluxed with alcohol, and after cooling was filtered off and washed with alcohol. The alcohol dissolved 216 mg. of non-volatile matter, which was not further investigated. The alcohol-insoluble material (308 mg.) had C, 47·7; H, 9·22; N, 7·55 %.

Some of this material was benzoylated by the method of Fischer [1900]. A 15 % yield of crystalline benzoylated product was obtained, which on recrystallization from water had M.P. 127°, not depressed by admixture with authentic benzoyl-*l*-valine, for which Karrer & van der Sluys Veer [1932] record M.P. 127°. Karrer & Schneider [1930] record benzoyl-*l*-*nor*valine as having M.P. 95°, and as forming a hydrate of M.P. 64°.

$68_c 13_w 21_m$. This fraction crystallized readily from a small volume of chloroform. The crystals, after filtering off and washing with chloroform, weighed 594 mg. and had M.P. 156°, which was not depressed on admixture with authentic acetyl-*l*-valine. $[\alpha]_D^{23^\circ} + 2{\cdot}5^\circ$ (alcohol, $c = 2$). (Synge [1939, 1] records acetyl-*l*-valine as having M.P. 157–158°. $[\alpha]_D^{20^\circ} + 5{\cdot}8^\circ$.) (Found: C, 52·7; H, 7·84; N, 8·3 %. Calc. for $C_7H_{13}O_3N$: C, 52·8; H, 8·17; N, 8·8 %.) A further 19 mg. of the same material were obtained on crystallization of the material in the mother liquors from chloroform-ether. No further crystals resulting, the material in the mother liquors was hydrolysed with H_2SO_4, freed from $SO_4^=$ with $Ba(OH)_2$ and extracted with alcohol as described for the previous fraction. The resulting alcohol-insoluble material (104 mg.) had C, 50·4; H, 8·89; N, 9·3 %. Valine

$C_5H_{11}O_2N$ has C, 51·2; H, 9·41; N, 11·98 %. A 1 % solution in water showed no optical activity in a 2 dm. tube.

The alcohol extract yielded 302 mg. of non-volatile matter which did not crystallize readily, and has not been further investigated.

$68_c13_w21_c$. This fraction gave no crystals from chloroform, but crystallized slowly from moist ether. The product, on recrystallization from moist chloroform ligroin, weighed 532 mg. (air-dry). It had M.P. 77°, and did not depress the M.P. of authentic acetyl-*l*-proline hydrate (see under fraction 68_c13_m).

The material in the mother liquors was hydrolysed and extracted with alcohol as previously described. Only a very small amount remained insoluble in alcohol, and neither this nor the alcoholic extract has been further investigated.

68_c13_m. This fraction yielded as crystals from chloroform a first crop of 473 mg., M.P. 183–184°, not depressed on admixture with authentic acetyl-*l*-leucine (cf. corresponding fraction from known mixture). $[\alpha]_D^{24^\circ}$ $-22{\cdot}6^\circ$ (alcohol, $c=3{\cdot}3$). (Found: C, 55·0; H, 8·94; N, 7·64 %. Calc. for $C_8H_{15}O_3N$: C, 55·5; H, 8·67; N, 8·1 %.)

The mother liquors from this material, by successive crystallization from ligroin and moist ether-ligroin, yielded 615 mg. of material having M.P. 78° (115° after drying *in vacuo*). $[\alpha]_D^{22^\circ}$ $-114{\cdot}0^\circ$ (water, $c=4{\cdot}2$) (rotation calculated for anhydrous compound). Du Vigneaud & Meyer [1932, 1, 2] record for acetyl-*l*-proline M.P. 81–82° (hydrate), 116–117° (anhydrous) $[\alpha]_D^{27^\circ}$ $-106{\cdot}7$, $-107{\cdot}1^\circ$ (water, $c=0{\cdot}5$). (Found: C, 52·9; H, 7·02; N, 8·62 %. Calc. for $C_7H_{11}O_3N$: C, 53·5; H, 7·01; N, 8·91 %.)

A second crop of the same material (M.P. 77°), weighing 203 mg., was obtained.

After this removal of acetylproline, it was possible to obtain a further crop of acetylleucine from a small volume of chloroform. 181 mg. $[\alpha]_D^{21^\circ}$ $-14{\cdot}5^\circ$ (alcohol, $c=1$). M.P. 155–169°. (Found: C, 55·5; H, 8·92; N, 6·4 %.)

The small amount of material still remaining in the mother liquors has not been further investigated.

$68_c13_c7{\cdot}5_w$. This fraction crystallized readily from water, and seemed nearly completely crystalline, although the later crops crystallized very slowly.

Crop I (852 mg.) had M.P. 175–179°. $[\alpha]_D^{20^\circ}$ $-13{\cdot}0^\circ$ (alcohol, $c=1{\cdot}5$). (Found: C, 55·2; H, 8·63; N, 6·6 %.)

Crop II (176 mg.) had M.P. 143–158°. $[\alpha]_D^{20^\circ}$ $-4{\cdot}5^\circ$ (alcohol, $c=1{\cdot}3$). It contained 4 % of incombustible residue, and after 'correction' for this, had elementary composition: C, 54·2; H, 8·13; N, 7·0 %. The residue (275 mg.) has not been further investigated.

$68_c13_c7{\cdot}5_m$. Crystallization from chloroform, which was very slow, yielded 180 mg. of material having M.P. 179–181° (crop I). $[\alpha]_D^{20^\circ}$ $-8{\cdot}4^\circ$ (alcohol, $c=1$). (Found: C, 54·8; H, 8·50; N, 7·6 %.)

Crystallization of the residue in the mother liquor from chloroform-ligroin gave crystalline material melting at 133–148° (crop II). Repeated crystallization of this yielded crystals (34 mg.) having M.P. 153–163°. These were not further investigated. The remaining material of crop II had $[\alpha]_D^{20^\circ}$ $-1{\cdot}7^\circ$ (alcohol, $c=1$). (Found: C, 53·4; H, 7·87; N, 7·0 %.)

The material in the mother liquors from crop II was hydrolysed with 2*N* HCl at 100° for 3 hr., and on cooling the aqueous phase was separated from the oil (see under fraction 68_m) and evaporated to dryness several times with water. The residue was taken up in water, and brought to *p*H 6 with LiOH. The mixture was again evaporated to dryness, and extraction with alcohol yielded 38 mg. of insoluble material, which has not been further investigated.

$68_c13_c7{\cdot}5_c$. This fraction did not readily give crystals from chloroform. Crystallization from ether-ligroin, followed by recrystallization from chloroform, gave 536 mg. of material having M.P. 141–143°, whose M.P. was not depressed on admixture with authentic acetyl-*dl*-phenylalanine of the same M.P. Despite reports in the literature of higher M.P. (151° [Knoop & Blanco, 1925], 150–151° [du Vigneaud & Meyer, 1932, 1], 148° [Neuberger, 1938, 2]), we agree with Jackson & Cahill [1938] in having been unable to prepare synthetic acetyl-*dl*-phenylalanine of M.P. higher than 143°. $[\alpha]_D^{20^\circ} + 2{\cdot}4^\circ$ (alcohol, $c = 1{\cdot}7$). For acetyl-*l*-phenylalanine Jackson & Cahill [1938] record $[\alpha]_D^{25^\circ} + 47{\cdot}6^\circ$ (alcohol), while for the *d*-isomer du Vigneaud & Meyer [1932, 1] record a corresponding figure of -51°. (Found: C, 63·1; H, 6·20; N, 5·9%. Calc. for $C_{11}H_{13}O_3N$: C, 63·7; H, 6·28; N, 6·77%.)

A further crop of 112 mg. of the same material was obtained.

As the residue would give no more crystals, it was hydrolysed and neutralized with LiOH in the same manner as the previous fraction. Precipitation by alcohol from concentrated aqueous solution yielded 144 mg. of material having $[\alpha]_D^{20^\circ}$ $-4{\cdot}9^\circ$ (water, $c = 0{\cdot}8$). (Found: C, 60·1; H, 8·01; N, 7·6%.) This elementary composition is intermediate between that of phenylalanine and leucine (isomers) of which this material is presumably a mixture.

Isolation of hydroxyamino-acid fraction from wool hydrolysate

The aqueous solution resulting from the second extraction of the acetylated hydrolysate in the Neuberger apparatus was evaporated to dryness and the acetamino-acids were freed from salt by extraction with alcohol [cf. Synge, 1939, 2]. The product resulting from evaporation of the alcohol was refluxed for 4 hr. with 450 ml. of 3 *N* H_2SO_4. This solution, when cool, was treated with 75 g. of phosphotungstic acid at a final volume of a litre. After storing for 15 hr. the precipitate was filtered off, and washed with a 1% solution of phosphotungstic acid in 1% H_2SO_4. The precipitate was found (by difference) to contain 29·3% of the total protein-N.

The combined filtrate and washings from this were treated with $Ba(OH)_2$ at 100° until just permanently alkaline to thymol blue. The resulting precipitate was thoroughly extracted with hot water, being maintained at the same *p*H by addition of $Ba(OH)_2$ when necessary; the filtrate and washings were then combined, and concentrated to a medium syrup. This was subjected to the acetylation-benzoylation procedure described by Synge [1939, 3], employing for the acetylation 200 ml. of 2 *N* NaOH and 20 ml. of acetic anhydride, and for the benzoylation 185 ml. of 2 *N* NaOH and 23 ml. of benzoyl chloride.

After the saponification of the *O*-benzoyl groups by $Ba(OH)_2$ as described Ba was removed exactly before the chloroform extraction, after which the aqueous layer was concentrated to dryness, giving the hydroxyamino-acids as their *N*-acetyl derivatives.

This fraction contained 5·9% of the protein-N (5·6% amino-N), in good agreement with the results obtained on wool by Synge [1939, 3] without previous removal of the higher monoamino-acids. The *N*-acetyl-hydroxyamino-acids formed a nearly colourless glass, which showed no sign of crystallization after storage in a desiccator for 6 months.

In a forthcoming paper we describe the further investigation of this fraction.

Isolation of the hydroxyamino-acids by acetylation-benzoylation after removal of the higher monoamino-acids by extraction of their acetyl derivatives into chloroform appears to be preferable to the direct procedure, since it minimizes the possibility of contamination of the fraction by monoacetaminoacids, which pass readily into chloroform from strong salt solution during the extraction of the *N*-acetyl-*O*-benzoyl-hydroxyamino-acids, but may not be so readily extractable by chloroform from the salt-free solution resulting from the saponification of the *O*-benzoyl derivatives.

DISCUSSION

The valine fractions. Abderhalden & co-workers [1930–37] have reported the occurrence of *l*-*nor*valine with valine in globin, casein, tussore silk, steer-horn and yeast proteins. We have obtained no positive evidence for its occurrence in wool, about two-thirds of the possible valines in fractions $68_c 13_w 21_w$ and $68_c 13_w 21_m$ having been identified as *l*-valine in the form of its acetyl and benzoyl derivatives.

The leucine fractions. The rotations and M.P. of the various preparations isolated having the composition of acetylleucine are summarized in Table 14.

Table 14. *Acetylleucine isomers from wool*

Fraction	Crop	Wt. (mg.)	M.P.	$[\alpha]_D$ (alcohol)
$68_c 13_m$	I	473	183–184°	$-22{\cdot}6°$
	II	181	155–169°	$-14{\cdot}5°$
$68_c 13_c 7{\cdot}5_w$	I	852	175–179°	$-13{\cdot}0°$
	II	176	143–158°	$-4{\cdot}5°$
$68_c 13_c 7{\cdot}5_m$	I	180	179–181°	$-8{\cdot}4°$
	II	34	153–163°	—
	III	241	133–148°	$-1{\cdot}7°$

From the data provided by Synge [1939, 1] it may be inferred that acetyl-*l*-leucine has M.P. 186–188°, $[\alpha]_D -23{\cdot}2°$ (alcohol), and acetyl-*l*-*iso*leucine, M.P. 150–151°, $[\alpha]_D +15{\cdot}6°$ (alcohol).

Acetyl-*l*-leucine does not on admixture depress the M.P. of acetyl-*l*-*iso*leucine or of acetyl-*dl*-leucine. Acetyl-*dl*-leucine on admixture depresses the M.P. of acetyl-*l*-*iso*leucine. It seems therefore unnecessary to postulate the occurrence of *nor*leucine in wool in order to account for the M.P. and rotations of the acetylleucine fractions isolated. Acetyl-*d*-*nor*leucine [Synge, 1939, 1] has the low M.P. 112–114° and an insignificant rotation in alcohol.

It has been shown in the control experiment described above that the procedure employed racemizes leucine to the extent of about 15 %. In the case of *l*-*iso*leucine, a corresponding epimerization to *d*-*alloiso*leucine may be expected. Since *d*-*alloiso*leucine has $[\alpha]_D -14{\cdot}2°$ (water), $-36{\cdot}8°$ (20 % HCl) [Ehrlich, 1907], which is nearly equal and opposite to that of *l*-*iso*leucine ($[\alpha]_D +11{\cdot}3°$ (water), $+37{\cdot}4°$ (20 % HCl) [Locquin, 1907]), it seems likely that the same may be true for the corresponding acetyl derivatives. Assuming this to be the case, and that 15 % racemization (epimerization) has occurred, the calculated leucine : *iso*leucine ratio in the isolated crops of acetylleucine isomers would be 4 : 1. The proportion of *iso*leucine left in the mother liquors would be greater than this, on account of the greater solubility of its acetyl derivative.

The phenylalanine fraction. No isolation of phenylalanine has been previously reported from wool, although Block [1939] reports a colorimetric determination, giving the phenylalanine-N as 2·2 % of the total N of the wool. Abderhalden & Voitinovici [1907] obtained in the ester fraction in which phenylalanine was to be expected a product having the elementary composition of leucine. In the present work we have isolated 1·32 % of the N of wool as a product agreeing in its properties with acetyl-*dl*-phenylalanine and having only

4·7 % of the optical activity of acetyl-*l*-phenylalanine. Moreover, since acetyl-*l*-phenylalanine is less soluble and crystallizes more readily than the racemic compound, there is no reason to suspect significant quantities of it in the mother liquors. The possibility of extensive racemization in the course of acetylation may be ruled out, since the procedure employed gave, in the hands of du Vigneaud & Meyer [1932, 1], a 92 % yield of acetyl-*l*-phenylalanine from *l*-phenylalanine, and we can confirm this from our own experience. Moreover, the other amino-acids in the same hydrolysate were not seriously racemized.

It does not seem to us necessary to postulate the occurrence of phenylalanine residues in wool other than in the *l* form. Levene & Steiger [1930] have shown that treatment of *d*-phenylalanine for 36 hr. at 100° with 20 % HCl induces no detectable racemization, whereas in glycyl-*d*-phenylalanine and the corresponding diketopiperazine up to 20 % racemization of the phenylalanine was observed under the same conditions. Little, if any, other work has been published on the racemization of amino-acid residues during the treatment of peptides etc. with mineral acids, although the question has assumed importance in connexion with the recent controversy over the occurrence of partially racemic glutamic acid in tumour hydrolysates. The earlier stages of this controversy have been reviewed by Pirie [1939; cf. also Graff *et al.* 1940, and Chibnall *et al.* 1940]. These last workers observe: 'Protein chemists have so far given but little attention to the occurrence of racemized amino-acids in protein hydrolysates, and it may well be that they are more widespread than has been imagined, for the possibility that racemization accompanies the splitting of certain peptide bonds during hydrolysis of the protein molecule must not be lost sight of.'

It seems to us possible that the position occupied by the phenylalanine residues in wool may be such as to render them more liable than other residues to racemization in the course of acid hydrolysis. In this connexion it is interesting that Jensen & Evans [1935], by subjecting insulin to treatment with phenyl*iso*cyanate, followed by acid hydrolysis, isolated a yield of the phenylhydantoin of *dl*-phenylalanine which suggested that a substantial proportion of the amino groups of this protein belong to phenylalanine residues.

It is possible that the present phenomenon may not be restricted to wool: Sharp [1939] isolated very considerably racemized phenylalanine from a myosin hydrolysate by ester distillation, and Baptist & Robson [1940] have commented on the high degree of racemization recorded in the literature for phenylalanine preparations from proteins.

Summary of present analysis

In Table 15 we summarize our analytical data on the seven end-fractions which we have investigated. In this table, each amino-acid is expressed in N as % of total N of the wool. Under each amino-acid col. I records material isolated and formally identified, while col. II refers to 'unidentified N'. In the cases of methionine and proline, these figures are based respectively on the 'volatile iodide' and 'non-amino-N' figures for each fraction. In the cases of valine, leucine isomers and phenylalanine, the figures in col. II are based on the residual amino-N of the fraction, and are allocated, in the case of fraction $68_C 13_C 7{\cdot}5_C$, on the basis of the elementary composition of the material isolated by acid hydrolysis from the mother liquors of acetylphenylalanine.

The total N of the end-fractions (17·87 % of total N) is in good agreement with the N figure for fraction 68_C (18·2 %), showing that the loss of material in the course of analysis is slight.

Table 15. *The higher monoamino-acids of wool*

(N as % of total N)

Fraction	N of fraction	Methionine		Valine		Proline		Leucines		Phenyl-alanine	
		I	II	I	II	I	II	I	II	I	II
$68_c13_w21_w$	1·66	—	0·31	0·17	0·92	-	0·26	—	—	—	—
$68_c13_w21_m$	2·81	--	0·09	2·01	0·16	—	0·55	--	—	—	—
$68_c13_w21_c$	1·72	—	0·01	—	0·13	1·29	0·16	·	0·13	—	—
68_c13_m	4·44	—	—	--	—	1·97	0·70	1·60	0·17	--	—
$68_c13_c7{\cdot}5_w$	3·17	—	—	—	--	-	—	2·52	0·65	—	—
$68_c13_c7{\cdot}5_m$	1·55	—	--	-	—	—	—	1 11	0·44	--	—
$68_c13_c7{\cdot}5_c$	2·52	—	—	—	—	—	-	-	0·58	1·32	0·62
Total	17·87	—	0·41	2·18	1·21	3·26	1·67	5·23	1·97	1·32	0·62
Combined totals		0·41		3·39		4·93		7·20		1·94	

In Table 16 the present analysis is correlated with the more important published data on the amino-acid composition of wool. In this table the amount of each amino-acid is expressed as N in % of total N of wool. Where authors have not recorded the N content of the wool analysed, it has been assumed to be 16·75 %. Barritt [1928] found the N content of a very wide variety of white wools to lie between 16·50 and 17·07 %.

The high amino-N content of the hydroxyamino-acid fraction from wool makes the presence of hydroxyproline unlikely.

This is in agreement with the failure of Abderhalden & Voitinovici [1907] to isolate hydroxyproline from wool or from sheep-horn where they made a special search for it.

Table 16. *Summarized data on the amino-acid composition of wool*

(N as % of total N)

Author ...	Abderhalden & Voitinovici [1907]	Marston [1928]	Block [1939]	Other authors		Present investigation
N content of wool %	—	17·8	15·4	—		—
Glycine	0·65	—	7·87	—		—
Alanine	4·13	—	—			
Valine	2·0	—	—	—		3·4
Leucine isomers	7·35	—	—	—		7·2
Phenylalanine	—	—	2·2	—		1·9
Proline	3·20	—	—	—		4·9
Methionine	—	—	—	0·3	Barritt [1934]	0·4
Cystine	5·08	8·59	9·93	—		—
Serine, etc.	0·05	—	—	7·5	Martin & Synge (to be published)	—
Threonine	—	—	—	4·7		—
Tyrosine	1·34	2·08	2·26	—		—
Tryptophane	—	1·39	0·62	—		—
Aspartic acid	1·45	—	—	4·56	Speakman & Townend [1937]	—
Glutamic acid	7·34	—	—	8·68		—
Ammonia	—	6·74	—	8·18		—
Arginine	—	18·42	18·16	20·2	Vickery [1940]	—
Histidine	—	10·50	1·23	—		—
Lysine	—	5·02	3·12	—		—

It will be seen from Table 16 that more than 90 % of the N of wool has now been reasonably accounted for in the form of ultimate hydrolytic products. The corresponding figure for gelatin, based on the figures of Dakin [1920], as modified by Bergmann & Stein [1939] for glycine and proline and by Synge [1939, 3] for hydroxyamino-acids other than hydroxyproline, is 92·5 %.

The serine and threonine values given in Table 16 are taken from our forthcoming paper.

Criticism of the new analytical procedure

In appraising the analytical value of the technique described in this paper, we wish to emphasize that the procedure is still in a crude state and is capable of technical development. Thus it is clear, from the consistent change of rotation of successive acetylleucine fractions shown in Table 14, that a longer extraction column, operating in a more controlled manner, could give practically complete separation of the components of this mixture.

It is natural to compare the present procedure with the Dakin butyl alcohol extraction and Fischer ester distillation procedures, to which its two stages are directly analogous, and which are the only methods for separating this group of amino-acids in general use among protein chemists.

The present procedure is not suited to deal with glycine or alanine. For methionine, valine, proline, leucine isomers and phenylalanine it has definite advantages. The procedure leading to fraction 68_C gives these amino-acids in 95% yield, and substantially free from amino-acids of other groups. Butyl alcohol extraction, according to Sharp [1939], may give significant amounts of basic and dicarboxylic amino-acids together with the monoamino-acids. In subsequent fractionation, the present procedure leads to a strikingly better separation of valine from leucine than those recorded for ester distillation in the literature. It should, however, be remembered that modern distillation technique has so far not been applied to the Fischer distillation. The degrees of racemization induced by the two procedures seem to be similar. The acetamino-acids have the advantage over the free amino-acids of a lower tendency to form mixed crystals, and therefore may prove of use for 'isotope dilution' analysis [Rittenberg & Foster, 1940] where high purity is called for, irrespective of yield. The acetamino-acids have definite melting points, which is useful for rapid identification. The quantity of protein required for an analysis (10 g.) by the present procedure is much smaller than that required for an ester distillation. Finally, the residues from the first extractions may advantageously be used for the isolation of hydroxyamino-acids by acetylation-benzoylation, as described above, and it is possible that other analytical procedures would benefit from a preliminary removal of the higher monoamino-acids.

SUMMARY

1. The history of the development of purification processes based on liquid-liquid extraction is reviewed.

2. The possibility of separating certain amino-acids as their acetyl derivatives by extractional fractionation in a chloroform-water counter-current train is discussed. The physical chemistry of the separation is outlined, and some new experimental data are provided on the equilibria concerned.

3. The design, construction and operation of a 40-unit counter-current liquid-liquid extraction train is described, together with tests of the efficiency of this machine in separating acetamino-acids.

4. On this basis an analytical procedure for the determination and isolation of methionine, valine, proline, leucine isomers and phenylalanine in protein hydrolysates is developed, and is tested on a complex known mixture of amino-acids. The results show that the new procedure has some advantages over the use of butyl alcohol extraction (Dakin) followed by ester distillation (Fischer).

5. The new procedure is applied to the analysis of a wool hydrolysate.

6. The results of the present investigation are summarized together with the published information on the amino-acid composition of wool.

We wish to express our gratitude to the International Wool Secretariat for their generous provision of materials and apparatus; to Mr Horsfall, Miss Thornton and Mr Warburton for help in the preparation of the figures in this paper; and to Prof. A. C. Chibnall, Mr H. E. Daniels, Sir C. J. Martin and Mr B. H. Wilsdon for their advice, interest and encouragement.

REFERENCES

Abderhalden & Bahn (1930). *Ber. dtsch. chem. Ges.* **63**, 914.

—— —— (1937). *Hoppe-Seyl. Z.* **245**, 246.

—— & Heyns (1931). *Hoppe-Seyl. Z.* **202**, 37.

—— —— (1932, 1). *Hoppe-Seyl. Z.* **206**, 137.

—— —— (1932, 2). *Hoppe-Seyl. Z.* **209**, 27.

—— & Reich (1930). *Hoppe-Seyl. Z.* **193**, 198.

—— & Voitinovici (1907). *Hoppe-Seyl. Z.* **52**, 348.

Arnow & Opsahl (1940). *J. biol. Chem.* **133**, 765.

Baernstein (1932). *J. biol. Chem.* **97**, 663.

—— (1936). *J. biol. Chem.* **115**, 25.

Baptist & Robson (1940). *Biochem. J.* **34**, 221.

Barritt (1928). *J. Soc. chem. Ind., Lond.*, **47**, 69 T.

—— (1934). *Biochem. J.* **28**, 1.

Bergmann & Stein (1939). *J. biol. Chem.* **128**, 217.

Block (1939). *J. biol. Chem.* **128**, 181.

Cherbuliez & Wahl (1925). *Helv. chim. Acta*, **8**, 571.

Chibnall, Rees, Williams & Boyland (1940). *Biochem. J.* **34**, 285.

Cornish, Archibald, Murphy & Evans (1934). *J. industr. Engng Chem.* **26**, 397.

Dakin (1920). *J. biol. Chem.* **44**, 499.

Ehrlich (1907). *Ber. dtsch. chem. Ges.* **40**, 2538.

Evans (1934). *J. industr. Engng Chem.* **26**, 860.

Fischer (1900). *Ber. dtsch. chem. Ges.* **33**, 2370.

—— (1901). *Ber. dtsch. chem. Ges.* **34**, 433.

Graff, Rittenberg & Foster (1940). *J. biol. Chem.* **133**, 745.

Hunter & Nash (1932). *J Soc. chem. Ind., Lond.*, **51**, 285 T.

—— —— (1934). *J. Soc. chem. Ind., Lond.*, **53**, 95 T.

Jackson & Cahill (1938). *J. biol. Chem.* **126**, 37.

Jensen & Evans (1935). *J. biol. Chem.* **108**, 1.

Johnson (1940). *J. biol. Chem.* **134**, 459.

Karrer & Schneider (1930). *Helv. chim. Acta*, **13**, 1281.

—— & van der Sluys Veer (1932). *Helv. chim. Acta*, **15**, 746.

Knoop & Blanco (1925). *Hoppe-Seyl. Z.* **146**, 267.

Levene & Steiger (1930). *J. biol. Chem.* **86**, 703.

Lewis (1916). *J. industr. Engng Chem.* **8**, 825.

Locquin (1907). *Bull. Soc. chim. Fr.* (4), **1**, 600.

McCabe & Thiele (1925). *J. industr. Engng Chem.* **17**, 605.

Marston (1928). *Australia. C.S.I.R. Bull.* No. 38 (Melbourne).

Mueller (1923). *J. biol. Chem.* **56**, 157.

Neuberger (1938, 1). *Biochem. J.* **32**, 1435.

—— (1938, 2). *Biochem. J.* **32**, 1452.

Pirie (1932). *Biochem. J.* **26**, 1270.

—— (1939). *Chemical Society Annual Reports*, **36**, 350.

Ponchon (1921). *Tech. mod.* **13**. 20, 55.

Randall & Longtin (1938, 1–3). *J. industr. Engng Chem.* **30**, 1063, 1188, 1311.
—— —— (1939, 1–3). *J. industr. Engng Chem.* **31**, 908, 1181, 1295.
Rittenberg & Foster (1940). *J. biol. Chem.* **133**, 737.
Saal & van Dijck (1933). *Proc. World Petroleum Congress, Lond.*, **2**, 352.
Sharp (1939). *Biochem. J.* **33**, 679.
Smith & White (1929). *J. phys. Chem.* **33**, 1953.
Speakman & Townend (1937). *Nature, Lond.*, **139**, 411.
Synge (1939, 1–4). *Biochem. J.* **33**, 1913, 1918, 1924, 1931.
Thiele (1935). *J. industr. Engng Chem.* **27**, 392.
Tucker & Reid (1932). *J. Amer. chem. Soc.* **55**, 775.
Vickery (1940). *J. biol. Chem.* **132**, 325.
—— & Schmidt (1931). *Chem. Rev.* **9**, 169.
du Vigneaud & Meyer (1932, 1). *J. biol. Chem.* **98**, 295.
—— —— (1932, 2). *J. biol. Chem.* **99**, 143.

13. ENZYMIC PROTEOLYSIS

4. AMINO-ACIDS OF CASEIN PHOSPHOPEPTONE[1]

By M. DAMODARAN and B. V. RAMACHANDRAN

From the University Biochemical Laboratory, Chepauk, Madras

(*Received 18 November 1940*)

TRYPSIN-RESISTANT phosphopeptones from casein have been studied by Posternak [1927; 1928] and by Rimington [1927, 1, 2]. The former isolated three compounds which he designated 'lactotyrins' and showed them to be made up of 15 to 18 amino-acid units containing serine, glutamic acid, aspartic acid and *iso*leucine. Posternak's papers contain no experimental details, and although his conclusion that the P in casein exists in the form of phosphoserine has been confirmed by the isolation of this amino-acid by Lipmann [1933], the generally accepted view of the structure of phosphopeptone itself is that advanced by Rimington [cf. Jordan Lloyd & Shore, 1938], according to whom the enzyme-resistant polypeptide is made up of 9 hydroxyamino-acid units consisting of 3 mol. of hydroxyglutamic acid, 4 mol. of hydroxyaminobutyric acid and 2 mol. of serine. The discrepancies in the results obtained by these authors and the fact that Harington & Randall [1931] were unable to identify hydroxyglutamic acid as a constituent of casein prompted the reinvestigation of this problem.

To obtain a trypsin-resistant phosphopeptone of constant composition advantage was taken of the fact that peptic digestion of casein automatically causes the separation of an insoluble complex, the so-called 'paranuclein', which contains 50–60 % of the P of casein and only about 20 % of its N. After digesting the paranuclein till constancy of amino-N was reached, the P-containing complex was precipitated with neutral lead acetate, redigested with trypsin and the phosphopeptone finally separated as the Ba salt insoluble in 50 % alcohol. Preparations so obtained were constant in composition and unaffected by further treatment with trypsin. The amino-N value of 10 % and the atomic N/P ratio of 3·2 to 3·3 indicated a polypeptide of 10 amino-acids attached to 3 phosphoric acid residues. N distribution determined according to Damodaran [1931, 1] gave amide-N values varying from 10 to 13 % in different analyses, dicarboxylic acid-N 43–47 % and monoamino-N 43–45 %, and a complete absence of basic and non-amino-acids. For isolation of the amino-acids present a combination of the butyl alcohol [Dakin, 1918], the Ba-alcohol [Kingston & Schryver, 1924] and the copper salt [Damodaran, 1931, 2] methods were used. Serine, *iso*leucine and glutamic acid were found to be the only amino-acids present. It was also seen early in the investigation that the glutamic acid isolated accounted for less than two-thirds of the dicarboxylic acid-N as found by N-distribution. In view of the previously reported presence in phosphopeptone of dicarboxylic acids other than glutamic acid and of hydroxyamino-acids other than serine, it was decided to determine hydroxyamino-acids and dicarboxylic acids by indirect methods in all amino-acid fractions separated from the hydrolysate.

For the identification of the hydroxyamino-acids oxidation with chloramine-T and subsequent preparation of suitable osazones of the hydroxy-aldehydes formed [Dakin, 1916; 1917] was made use of. The *p*-nitrophenyl- and the 2:4-dinitrophenyl-osazones of glyoxal (from serine), of methylglyoxal (from hydroxyaminobutyric acid) and of malic semi-aldehyde (from hydroxyglutamic acid) have M.P. ranging between 299 and 319° and are therefore unsuited for identification; the phenylosazones, on the other hand, of methylglyoxal and glyoxal, though less insoluble, have M.P. 145 and 167° respectively, which make them useful for differentiation. Figures are not available for the M.P. of the phenyl- and the 2:4-dinitrophenyl-osazones of malic semialdehyde. By the use of these derivatives it was found that there was no evidence for the presence of any hydroxyamino-acid other than serine. As quantitative isolation of this amino-acid was naturally difficult the insolubility of the 2:4-dinitrophenyl-osazone formed from it was made use of for quantitative estimation.

---

[1] First appeared as a note in *Nature, Lond.*, 145, 857

For the indirect estimation of dicarboxylic acids at various stages of separation the titration method of Harris [1929] was used. It soon became obvious that the amount of glutamic acid isolated was much less than the amount indicated either by the Foreman method or by titration. This discrepancy, which could not be accounted for by losses in isolation, was finally found to be caused in the case of the Foreman method by the precipitation of serine along with glutamic acid and in the titration method by the presence of ether-extractable non-nitrogenous fatty acids. From the precipitate obtained by treatment with $Ca(OH)_2$ or $Ba(OH)_2$ and alcohol according to Foreman glutamic acid and serine could be separated by fractional crystallization. Further, working with pure serine it was found that from a 3·3 % solution treated by this method this amino-acid is precipitated to the extent of 63·8 %. In the titration method the high values for dicarboxylic acid obtained were eliminated if the hydrolysate were first submitted to prolonged extraction with ether, the residual titratable acidity being then equivalent to the glutamic acid that could be isolated.

The ether-extractable acidic material, which was free from nitrogen, was found to be a mixture, the main component of which was glyceric acid, the latter being isolated and identified as its quinine salt. Varying amounts of lactic and pyruvic acids were also usually present, the quantities of the two being determined by the methods of Leib & Zacherl [1932] and of Simon & Neuberg [1931] respectively. The nature of the non-nitrogenous fatty acids indicated their secondary origin in the decomposition of serine [Abderhalden & Broich, 1933], and suggested that the NH_3 formed during acid hydrolysis of the phosphopeptone also had the same origin. The amide-N did in fact vary in different analyses; on following the time course of NH_3 liberation during hydrolysis by dilute acid according to Vickery [1922] it was seen that the NH_3 did not arise from an amide group but from deamination. The equivalence of the ether-extractable acidity to the NH_3 present in any particular hydrolysate left no doubt that the NH_3 was formed by the secondary decomposition of serine.

The results of the analyses by direct and indirect methods are summarized in Table 1.

Table 1. *Amino-acid composition of phosphopeptone*

	N as % of total N	
Amino-acid	By isolation	By estimation
Glutamic acid	29·70	29·97
*iso*Leucine	27·90	—
Serine	13·10	28·90
Ammonia	—	10·90

The hydrolysate is seen to consist of 30 % each of the three amino-acids, serine, glutamic acid and *iso*leucine. Assuming, as we are justified in doing, that the NH_3 formed during hydrolysis represents decomposed serine, it is concluded that *the phosphopeptone is a polypeptide of 10 amino-acids made up of 4 mol. of serine and 3 mol. each of glutamic acid and isoleucine.*

EXPERIMENTAL

Materials and methods

Casein was prepared (from reconstituted milk made with Australian skimmed milk powder) by the method of Cohn & Hendry [1930] and contained 15·6 % N and 0·8 % P. B.D.H. pepsin and trypsin were used for digestion.

The following methods were frequently made use of:

Oxidation of the fractions with chloramine-T and preparation of the osazones	Dakin [1918] and Gulland & Morris [1934] with modifications
Titration of dicarboxylic amino-acids	Harris [1929]
Determination of lactic acid	Leib & Zacherl [1932]
Determination of pyruvic acid	Simon & Neuberg [1931]
Organic and inorganic P	Fiske & Subbarow [1925]

Preparation of enzyme-resistant phosphopeptone

(*a*) *Preparation of 'paranuclein'.* 5 kg. of wet freshly precipitated casein, corresponding to about 1 kg. dry weight, were uniformly dispersed in 15 l. of $N/10$ H_2SO_4 at 37°, the *p*H adjusted to 1·8 by the addition of 5 N H_2SO_4 and the mixture incubated under toluene after addition of 25 g. of pepsin dissolved in 100 ml. of $N/10$ H_2SO_4. After 48 hr. the *p*H, which had risen to 2·2, was brought back to 1·8 and a further 25 g. of pepsin added. Amino-N reached constancy at about 20 % in 7 days. No inorganic P was formed at this stage in the digest. From the paranuclein formed as a compact precipitate at the bottom most of the clear yellow supernatant liquid was siphoned off and the remaining suspension was poured into large fluted filters and allowed to drain overnight in the refrigerator. The pasty solid was collected, stirred up with large volumes of water and washed free from H_2SO_4 by decantation and filtration as before. The product obtained after drying contained 15·2–15·6 % N, about 2 % P and had an atomic N/P ratio of 15–18 (compared with a ratio of about 44 for casein). Amino-N by the Van Slyke method was 12 %. The usual yield of dry paranuclein from 1 kg. of casein was 200–220 g., representing about 20 % of the N and 50–60 % of the P of casein.

(*b*) *Digestion of paranuclein with trypsin.* Trial experiments showed that digestion with trypsin at *p*H 8·3 caused only minimal liberation of inorganic P. At the end of 7 days when tryptic hydrolysis had reached completion (at 50–52 % of total hydrolysis) the inorganic P present was less than 5 %. The wet paranuclein from the peptic digestion was suspended in about 3 l. of distilled water and N NaOH added slowly with vigorous mechanical stirring till solution was complete. The solution, the *p*H of which was usually about 7 at this stage, was brought to 8·3 by the further addition of NaOH and incubated at 37° under toluene with 5 g. of trypsin. After 24 hr. amino-N of the solution was 40 % of the total N and the *p*H 6·3. No inorganic P was present. After readjusting the *p*H and adding a further 5 g. of trypsin, digestion was allowed to continue till amino-N failed to increase. This happenod usually in 7 days when amino-N was about 53 % and about 5 % of the P was present in inorganic condition.

(*c*) *Precipitation of phosphopeptone as Ba salt.* The digest was now brought to *p*H 4·6 with glacial acetic acid and after keeping overnight in the ice-chest was filtered from the small amount of suspended material. To the filtrate a concentrated solution of neutral Pb acetate was added with vigorous stirring till precipitation was complete. The Pb precipitate, which contained the whole of the organic P of the digest, was separated by centrifuging, suspended in water and decomposed by H_2S. The filtrate (about 1·5 l.), freed from Pb and H_2S, was redigested with 2 g. of trypsin and reprecipitated with Pb acetate as before. The solution obtained after decomposing the precipitate and freeing from H_2S was treated with 30 g. of Ba acetate, made alkaline with $Ba(OH)_2$ and rapidly filtered into a beaker containing acetic acid. After adjusting the *p*H to 7 by the addition of $Ba(OH)_2$ an equal volume of alcohol was added with stirring. The fine precipitate obtained was separated on the centrifuge, washed repeatedly with 50 % alcohol, then with absolute alcohol and ether and finally dried *in vacuo* over H_2SO_4.

Redigestion of the Pb precipitate with trypsin was found to be an essential condition for obtaining a Ba salt of constant composition which was resistant to further enzyme action. In earlier experiments in which the decomposed Pb salt was directly converted into the Ba compound, amino-N values ranging from 7·96 to 9·72 % and N/P ratios varying from 2·6 to 3·72 were obtained. However, preparations obtained as described above showed little variation in composition, as will be seen from the analyses of different samples (Table 2).

Table 2. *Analyses of Ba phosphopeptones*

Preparation no.	Amino-N as % of total N	N %	P %	N/P atomic	Ba %	Opt. rotation $[\alpha]_D^{31°}$ in H_2O
III	9·94	6·48	4·53	3·20	33·45	+34·81°
IV	9·84	6·44	4·26	3·35	33·12	
V	9·98	6·39	4·58	3·16	34·02	
VI	9·96	6·46	4 34	3·30	33·31	

Properties of the phosphopeptone

The Ba salt was extremely soluble in water, giving a water-clear solution which was neutral in reaction. The free peptide obtained by removing the Ba quantitatively with H_2SO_4 was strongly acidic. It was not precipitable by trichloroacetic, phosphotungstic or tannic acid, but was precipitated by heavy metal salts such as Pb, Cu and U acetates. It gave none of the usual colour reactions for individual amino-acids and was free from inorganic P. It underwent no hydrolysis on treatment with trypsin or intestinal erepsin. The whole of the P was split off by 1 % alkali in 24 hr. When an aqueous solution, made alkaline with $Ba(OH)_2$, was kept at 37° for a few hours and filtered from the precipitated Ba phosphate, the filtrate after being quantitatively freed from Ba was slowly acted upon by trypsin, amino-N increasing from 10 to 25 % in the course of a week. The resistance of the phosphopeptone to enzyme action is thus obviously due to the presence of the phosphoric acid groups.

Nitrogen distribution

N-distribution was determined according to Damodaran [1931, 1]. The results are given in Table 3.

Table 3. Distribution of N in the phosphopeptone

	Nitrogen	
	mg.	%
Total N	171·10	100·00
Humin-N	0·00	0·00
Ammonia-N	18·65	10·90
Dicarboxylic acid-N	77·59	45·37
Monoamino-N	73·41	42·90
Basic-N	0·00	0·00
Non-amino-N	0·00	0·00
Total	169·65	99·17

Separation of amino-acids in the phosphopeptone (scheme I)

Outline. The phosphopeptone was hydrolysed with H_2SO_4 and the hydrolysate (A), after being freed from H_2SO_4, phosphoric acid and NH_3, was treated with $Ba(OH)_2$ and alcohol. From the precipitate (B), after removal of Ba quantitatively, glutamic acid (Bi) and serine (Bii*a*) were obtained by fractional crystallization. A small amount of uncrystallizable syrup was left. The filtrate (C) from the Ba-alcohol precipitate, after being freed from Ba quantitatively, gave on fractional crystallization two or three crops of *iso*leucine (Ci) and finally serine (Cii*a*).

In all the fractions serine was determined by conversion into the dinitrophenylosazone of glyoxal and the apparent dicarboxylic acid content by titration according to the method described by Harris [1929] for the determination of unbalanced carboxyl groups in dicarboxylic monoamino-acids.

Hydrolysis. 15 g. of the Ba salt of the phosphopeptone were dissolved in 37·5 ml. of water, 14 ml. of conc. H_2SO_4 added and the mixture boiled under reflux for 18 hr. After making up to volume and removing an aliquot for analysis, H_2SO_4 and phosphoric acid were removed by means of $Ba(OH)_2$, the former at neutrality to Congo red and the latter at strongly alkaline reaction. The $BaSO_4$ and the Ba phosphate contained respectively 5·23 and 1·0 % of the total N. The alkaline filtrate from the Ba phosphate was concentrated *in vacuo* at 40° to about 1/5 vol. (about 20 ml.) for the removal of NH_3 and treated with 200 ml. of alcohol little by little with vigorous shaking to precipitate the dicarboxylic acids. The mixture was centrifuged and the sticky precipitate washed twice with 20 ml. portions of 95 % alcohol. The precipitate was dissolved in 20 ml. of hot water and after adding a small amount of $Ba(OH)_2$ was reprecipitated by the addition of 200 ml. of alcohol. The precipitate (B) was dissolved in water and made up to volume. It contained 47·7 % of the total N of the hydrolysate. The filtrate (C) from the Ba-alcohol precipitate was freed from alcohol by evaporation *in vacuo* and worked up as described later.

Dicarboxylic acid fraction (B). The solution was quantitatively freed from Ba, the $BaSO_4$ thoroughly washed with boiling water and the washings added to the main fraction. A portion of the solution was oxidized with chloramine-T and the serine-N was found to be 33·18 % of the N of this fraction. Titration of another portion of the solution showed an acidity corresponding to 100·89 % of dicarboxylic acid-N. The main solution was concentrated in the desiccator when thick prismatic crystals were obtained. The first crop weighed 1·8 g., while a second crop of 0·5 g. was obtained after further concentration. The N in these fractions amounted to 29·7 % of the total N of the hydrolysate and 63 % of the N of the Ba-alcohol precipitate. The two crops were combined (Bi) and recrystallized from water and the substance was identified as glutamic acid having N 9·53 %, amino-N 9·57 % (for glutamic acid, N 9·56 %) and M.P. 199°, which was not lowered by admixture with pure glutamic acid. 41 mg. of the acid required 2·82 ml. of 0·98 $N/10$ NaOH for neutralization to bromothymol blue, giving mol. wt. 148·4 (calc. 147·0). By saturating a concentrated solution of the substance with HCl gas in the cold it was quantitatively converted into glutamic acid hydrochloride with M.P. 213° and N 7·57 % (calc. for glutamic acid hydrochloride, N 7·63 %, M.P. 214°).

The mother liquor from the glutamic acid (fraction *B*ii) contained 0·1322 g. of N, of which 88 % was serine-N, and had a titratable acidity corresponding to 109 % of dicarboxylic acid-N. By treatment with alcohol as described in detail under the monocarboxylic acid fraction (*C*), 0·1 g. of crystalline amino-acid was obtained (*B*ii*a*). The needle-shaped crystals on recrystallization from aqueous alcohol yielded beautiful hexagonal plates melting at 228° and containing 13·23 % N (for serine, N 13·33 %, M.P. 228°). For further identification a portion of the substance was oxidized with chloramine-T; the resulting solution was divided into three portions and treated respectively with phenylhydrazine, *p*-nitrophenylhydrazine and 2:4-dinitrophenylhydrazine. The osazones obtained were recrystallized, the phenylosazone from aqueous alcohol and the nitrophenylosazones from hot nitrobenzene. A portion of serine was similarly oxidized and the osazones were prepared. The M.P. and the mixed M.P. are given in Table 4.

Table 4. *Melting points of osazones*

	M.P. of osazones		
	From *B*ii*a*	From serine	Mixed
Phenylosazone	167°	167·5	167°
p-Nitrophenylosazone	311	311°	311°
2:4-Dinitrophenylosazone	318°	319°	318°

The uncrystallizable mother liquor (*B*ii*b* containing 0·1109 g. N) contained 84·7 % of serine as determined by oxidation and was also strongly acidic. But persistent attempts failed to show the presence of a dicarboxylic acid in this fraction.

Monocarboxylic acid fraction (*C*). The filtrate from the Ba-alcohol precipitate contained 0·3054 g. N or 40·44 % of the total N of the hydrolysate. The solution was freed from Ba quantitatively and allowed to concentrate in the desiccator. A white amorphous material separated on the sides of the dish and on the surface of the liquid. The material was broken up and filtered and the mother liquor on further concentration gave two more crops of a similar substance. The N contents were 10·63, 10·68 and 10·98 % respectively. The first crop did not contain any serine-N, while the other two crops contained 3 and 10 % serine-N respectively. The crops were mixed together (*C*i) and recrystallized from water in which they were only sparingly soluble, when the same amorphous material as before was obtained (1·54 g.) but without contamination with serine. When the substance was dissolved in a small amount of hot water and alcohol was added to turbidity, needle-shaped crystals aggregated in bunches were obtained. The M.P. of the original amorphous material was 265°, while the crystals melted at 275°. The N content of 10·68 % indicated that the substance was one of the leucines.

For identification the substance was converted into its Cu salt. 1 g. of the material was dissolved in a small amount of water on the water bath and copper carbonate was added little by little till no more dissolved. The mixture was heated for about 30 min. on the water bath and filtered. The residue of copper carbonate was thoroughly washed with hot water till the washings were colourless. The combined filtrate and washings were evaporated to dryness on the water bath and the powder dried to constant weight at 100° in the air oven. It was repeatedly extracted by shaking with dry methanol freshly distilled over Ca, when almost the whole of the substance went into solution leaving a negligible residue. The methanol-soluble Cu salt was freed from alcohol by evaporation and recrystallized from water, from which it was obtained in the form of light blue needles. The crystals contained N 8·63 and Cu 19·63 % (calc. for Cu *iso*leucine, N 8·47 and Cu 19·51 %). A small amount of the Cu salt was decomposed by H_2S and the solution on concentration gave beautiful hexagonal plates of *iso*leucine melting at 275°. A 3·42 % solution of the substance in 20 % HCl had $[\alpha]_D^{31^\circ} + 26{\cdot}5^\circ$.

The mother liquor from Ci was heated on the water bath and alcohol was added drop by drop to turbidity. The sides of the dish were well scratched and the dish was left in a desiccator in the ice-chest overnight, when a mass of needle-shaped crystals was obtained. These were filtered quickly under centrifugal force, washed with alcohol and dried (Cii*a*). Another crop was obtained by concentration and addition of alcohol as before. The combined crops (0·448 g.) were recrystallized from aqueous alcohol. The hexagonal plates resembled fraction Bii*a* closely in all respects: N 13·32 %, M.P. 228°. The osazones were also identical with those of Bii*a*. The substance was therefore serine.

Table 5. *Analyses of fractions in scheme I*

		N of fractions		
	Fractions	In g.	As % of N of fraction	As % of T.N. of hydrolysate
A.	Hydrolysate	0·7554		100·00
	Dicarb.-N	0·3358		44·46
	Serine-N	0·2099		27·80
	Ammonia-N	0·0825		10·92
B.	Ba-alcohol precipitate	0·3603		47·70
	Dicarb.-N	—	100·89	-
	Serine-N	—	33·18	-
Bi.	*Glutamic acid* (2·3 g.)	0·2244	—	29·70
Bii.	Filtrate from (Bi)	0·1322	—	17·50
	Dicarb.-N	—	109·30	—
	Serine-N	—	88·00	—
Bii*a*.	*Serine* (0·1 g.)	0·0132	—	1·75
Bii*b*.	Mother liquor from above: uncrystallizable	0·1109	—	14·67
	Dicarb.-N	—	110·00	—
	Serine-N	—	84·72	—
C.	Filtrate from Ba-alcohol precipitate	0·3054	—	40·44
	Dicarb.-N	—	0·00	-
	Serine-N	—	34·72	-
Ci.	iso*Leucine* (1·54 g.)	0·1644	—	21·78
Cii.	Filtrate from above	0·1280	—	16·94
	Dicarb.-N	—	0·00	—
	Serine-N	—	82·56	—
Cii*a*.	*Serine* (0·45 g.)	0·0585	—	7·75
Cii*b*.	Uncrystallizable mother liquor	0·0629	—	8·33
	Dicarb.-N	—	0·00	—
	Serine-N	—	76·23	—

No crystals were obtained from the mother liquors Bii*b* and Cii*b* left from the dicarboxylic and the monocarboxylic acid fractions respectively. The total N in the fractions amounted to about 23 % of the hydrolysate-N. The fractions consisted mainly of serine, the amounts of which in the two fractions were 84·7 and 76·2 % respectively. They probably contained small amounts of the other amino-acids and also serine-decomposition products which prevented the amino-acids from crystallizing. On oxidation of the fractions only glyoxal osazones were obtained, which showed that serine was the only hydroxyamino-acid present in the hydrolysate. Conversion into Cu salts failed to show the presence of any aspartic acid. The analyses of the various fractions are given in Table 5.

Separation of the amino-acids in the phosphopeptone (scheme II)

Outline. The hydrolysed phosphopeptone (A) freed from H_2SO_4, phosphoric acid, NH_3 and Ba was acidified with H_2SO_4 and continuously extracted with ether for 48–72 hr. The H_2SO_4 was removed quantitatively and the solution after concentration was extracted with butyl alcohol 6–8 times. The butyl alcohol extracts (B) were concentrated *in vacuo* and the solids which crystallized out (Bi) were converted into the Cu salts. The Cu salts were separated into two fractions, Bi*a* soluble in methanol and Bi*b* insoluble in methanol. *iso*Leucine was isolated from the former and serine from the latter. The butyl alcoholic mother liquor (Bii) contained little nitrogenous material.

The aqueous solution (C), after extraction with butyl alcohol, was saturated with HCl gas and glutamic acid hydrochloride (Ci) was obtained. The filtrate from this (Cii), after removal of chloride, was concentrated and treated with alcohol to turbidity and cooled, when crystalline serine (Cii*a*) separated out. An uncrystallizable mother liquor (Cii*b*) containing 15·95 % of the total N was left.

Hydrolysis-ether extraction. 60 g. of the Ba salt were hydrolysed with H_2SO_4 and Ba, PO_4, SO_4 and NH_3 were removed as before. The $BaSO_4$ and Ba phosphate contained respectively 4·17 and 1·2 % of the total N. The solution was concentrated *in vacuo* to about 50 ml., acidified with about 5 ml. of $10N$ H_2SO_4 and extracted with ether in a continuous extractor for 72 hr., after which the dicarboxylic acid-N determined on an aliquot after quantitative removal of H_2SO_4 was found to be 30·0 % of the total N. Further extraction did not reduce the titratable acidity. The ether extract was evaporated in the presence of about 30 ml. of water and the aqueous solution was made up to a volume of 50 ml. It contained only 2·1 % of the total N but required 258 ml. of $N/10$ NaOH for neutralization, corresponding to a dicarboxylic acid-N value of 13·6 %.

*Butyl alcohol extraction—*iso*leucine as Cu salt.* The ether-extracted hydrolysate was quantitatively freed from H_2SO_4, concentrated to 100 ml. and extracted six times by shaking for 6 hr. each with 300 ml. portions of butyl alcohol. The butyl alcohol extracts were evaporated in three lots and the crystalline material which separated contained respectively 0·5723 g., 0·1168 g. and 0·0549 g. of N (5·45, 1·12 and 0·5 g. of solid respectively). The N contents were 10·51, 10·62 and 10·98 %. They showed no titratable acidity but contained respectively 9·61, 16·43 and 16·9 % of serine-N. The solids were combined and converted into the Cu salts as already described and separated into two fractions, one of Cu salts soluble in methanol (7·6 g.) and the other insoluble (0·98 g.). The methanol-soluble substance on recrystallization from water readily yielded crystalline Cu *iso*leucine: N 8·45 %; Cu 19·58 % (calc. for Cu *iso*leucine, N 8·47 and Cu 19·51 %). After removal of Cu by H_2S the amino-acid was obtained in pure condition with N 10·67 % and $[\alpha]_D^{32°} = +25·76°$ in 20 % HCl for a 3·88 % solution.

The methanol-insoluble, water-soluble Cu salt after decomposition with H_2S was found to consist chiefly of serine as determined by the oxidation method; it was therefore added to the next fraction (C).

The material extracted by butyl alcohol was almost completely crystallizable, the final mother liquor (B ii) containing only 3·4 % of the total N.

Glutamic acid hydrochloride. The aqueous solution, (C) after extraction with butyl alcohol (which contained 51·82 % dicarboxylic acid-N and 41·62 % serine-N), was concentrated to a small volume, cooled in ice and saturated with dry HCl gas. After 24 hr. in the refrigerator the separated glutamic acid hydrochloride (Ci) was filtered; from the concentrated filtrate a second crop was similarly obtained. The two fractions (9·18 g.) together contained 27·28 % of the total N. The N content of the mixed solid was 7·7 %. A portion of the substance was recrystallized by dissolving in water and saturating in the cold with dry HCl gas when pure crystals were obtained: N 7·63 %, M.P. 213°. 0·1100 g. of the substance gave 0·0867 g. of AgCl. The molecular weight of the substance was 181·8. The molecular weight of glutamic acid hydrochloride is 183·5.

Serine. The filtrate from the glutamic acid hydrochloride (Cii) was freed from HCl by repeated evaporation to dryness *in vacuo* with water and finally by means of Ag_2SO_4. After removal of H_2SO_4 quantitatively by means of $Ba(OH)_2$, serine was crystallized out by the addition of alcohol as described before. The mixed crops (Cii*a*) weighed 2·2 g. and contained 12·98 % N. A sample recrystallized from aqueous alcohol contained 13·23 % N and melted at 228°. 40 mg. of the substance gave 120·7 mg. of 2:4-dinitrophenylosazone, while the same

Table 6. *Analyses of fractions in scheme II*

	Fractions	N of fractions		
		In g.	As % of N of fraction	As % of total N of hydrolysate
A.	Hydrolysate	2·5460	—	100·00
	Dicarb.-N	0·7610	- -	29·97
	Serine-N	0·7358		28·90
	Ammonia-N	0·2774		10·90
B i.	iso*Leucine* (5·84 g.)	0·6229		24·46
B ii.	Butyl alcohol mother liquor	0·0860		3·38
C.	Aqueous fraction after butyl alcohol extraction	1·5561		61·12
	Dicarb.-N	- -	51·82	- -
	Serine-N	—	41·62	- -
Ci.	*Glutamic acid hydrochloride* (9·18 g.)	0·6942	-	27·28
Cii.	Filtrate from above	0·8467	-	33·25
	Dicarb.-N	—	10·45	—
	Serine-N	—	75·04	—
Cii*a*.	*Serine* (2·2 g.)	0·2796	—	10·98
Cii*b*.	Mother liquor from above	0·5671	- -	22·27
	Dicarb.-N	—	15·48	—
	Serine-N	—	62·74	- -
	Amino-acids from above by scheme I method			
	Glutamic acid (0·2 g.)	0·0191	-	0·75
	iso*Leucine* (0·69 g.)	0·0878	-	3·45
	Serine (0·4 g.)	0·0540	- -	2·12
	Final uncrystallizable syrup	0·4062	- -	15·95
	Dicarb.-N	—	16·95	—
	Serine-N	—	74·30	—

amount of serine gave 118·4 mg. of the osazone. The three osazones were prepared and the M.P. were identical with those of the osazones obtained from B ii *a* and C ii *a* in scheme I.

From the mother liquor (C ii *b*), by adopting the method of separation described in scheme I, 0·2 g. of glutamic acid, 0·69 g. of *iso*leucine and 0·4 g. of serine were obtained. The final mother liquor, which contained 15·95 % of the total N of the hydrolysate, was uncrystallizable. The results are summarized in Table 6.

Effect of ether extraction on the 'dicarboxylic acid-N' of hydrolysates

The hydrolysate obtained from 20 g. of the Ba salt, after determination of NH_3, was carefully freed from NH_3 and all reagents quantitatively made up to 100 ml. and portions were used for the following experiments. (i) In 2 ml. aliquots the apparent 'dicarboxylic acid-N' was determined by titration to *p*H 7 against *N*/10 NaOH. (ii) 20 ml. aliquots were acidified with 2 ml. of 5*N* H_2SO_4, continuously extracted with ether and the residual acidity determined by titration after removal of H_2SO_4 quantitatively. The ether extract was evaporated, the residue taken up in water and titrated to *p*H 7. (iii) In a 50 ml. aliquot glutamic acid was isolated as the hydrochloride. The results obtained, expressed as *N*/10 BaOH for the whole of the solution, were:

	ml. of *N*/10 NaOH
Total acidity	418·0
Acidity after extraction with ether	286·4
Acidity in the ether-extractable material	122·0
Glutamic acid as hydrochloride	268·7
Ammonia	128·3

The approximate equivalence of the acidity after ether extraction to the glutamic acid isolated and that of the ether-extractable acidity to the ammonia formed during the hydrolysis are patent.

Nature of the ether-extractable material

The fatty acids in the ether extracts did not consist entirely or even mainly of the acids observed by previous workers among the decomposition products of serine. Abderhalden & Broich [1933], who studied the decomposition of serine by H_2SO_4, obtained pyruvic acid, lactic acid, oxalic acid, glycine, alanine and NH_3, while Daft & Coghill [1931] obtained all the above-mentioned products except pyruvic acid during the decomposition of serine by alkali.

The aqueous solution from the ether extract, obtained as above, contained pyruvic acid, but estimation as the 2:4-dinitrophenylhydrazone [Simon & Neuberg, 1931] showed that only 15 % of the total acidity of the extract was due to this acid. Lactic acid estimated by the method of Friedemann *et al.* [1927], as modified by Leib & Zacherl [1932], was found to be present to the extent of 7 %. Thus the major portion of the mixture remained unaccounted for. The extract did not give any colour reactions for the common fatty acids. Ammoniacal $AgNO_3$ was not reduced. A brucine salt was prepared but could not be obtained crystalline. A crystalline quinine salt identical in properties with those of the quinine salt of *l*-glyceric acid described by Nef *et al.* [1917] was, however, obtained in good yield by the following procedure: the aqueous solution of the ether-extractable material was treated with a slight excess of 2:4-dinitrophenylhydrazine dissolved in an equal volume of 4*N* H_2SO_4 to remove pyruvic acid. The precipitated hydrazone was filtered off, and the filtrate was made alkaline with an excess of $Ba(OH)_2$ and filtered. The solution was extracted repeatedly with ethyl acetate to remove excess of the phenylhydrazine, boiled for a few minutes to remove ethyl acetate and freed from Ba quantitatively by H_2SO_4. The solution was concentrated *in vacuo* and treated on the water bath with an alcoholic solution of quinine drop by drop till the reaction was alkaline. After keeping on the water bath for about 30 min. the solution was cooled, and the excess of quinine was removed by extraction with ether. On concentration *in vacuo* a crystalline mass of the quinine salt was obtained, which was recrystallized from water.

The M.P. of the salt was 164° (165° given by Nef *et al.* [1917]), which was not affected by admixture with the quinine salt of authentic glyceric acid (prepared from serine by deamination with nitrous acid). The weight of the quinine salt obtained from the ether extract (0·35 g. from an aqueous solution requiring 12·6 ml. of *N*/10 NaOH for neutralization) suggests that the main fatty acid produced is glyceric acid. For a more exact determination of glyceric acid the method suggested by Rapoport [1935] was tried. According to this author serine is deaminated with nitrous acid to glyceric acid, which is then estimated from the blue colour formed on treatment with naphthoresorcinol dissolved in conc. H_2SO_4. In our experiments no blue colour was obtained with naphthoresorcinol either with the material from the ether extract or with serine itself treated according to the method described. Serine prepared in this laboratory from silk and a sample obtained from Hoffmann-la Roche were tried; the naphthoresorcinol was also obtained from the same firm.

As Friedemann *et al.* [1927] have shown that glyceric acid can give rise to lactic acid in their method, the lactic acid value mentioned previously does not provide conclusive evidence of the presence of this acid in the ether-extractable material.

The ether extract contained 2·1 % of the total N of the hydrolysate which, even if it was wholly a dicarboxylic acid, would not account for more than about 15 % of the total acidity. Oxidation of the ether extract with chloramine-T and treatment of the resulting solution with 2:4-dinitrophenylhydrazine did not give an osazone.

Table 7. *Time-course of NH_3 liberation during hydrolysis*

	NH_3 as % of total N	
Time in hr.	With 25 % H_2SO_4	With *N* HCl
1	—	0·34
2	—	1·05
4	—	1·60
6	7·88	1·89
8	8·73	2·70
12	9·80	3·97
18	10·92	5·91
24	15·20	6·86

Determination of serine by oxidation with chloramine-T

The method used was a modification of those of Dakin [1918] and Gulland & Morris [1934] for the estimation of 'hydroxyglutamic acid'.

5 ml. of a solution containing 40 mg. of serine (1 mol.) were treated with 0·11 g. of chloramine-T (1 mol.) and the mixture kept on a water bath (60–70°) for an hour. It was then cooled in ice, filtered and the precipitate washed with ice-cold water. The filtrate and the washings were diluted to about 40 ml. and 0·22 g. (3 mol.) of 2:4-dinitrophenylhydrazine dissolved in 40 ml. of warm 4 *N*

HCl was added. The mixture was kept in a boiling water bath for about an hour with occasional stirring, after which the precipitated osazone was filtered hot through a weighed sintered glass crucible and washed thoroughly, first with $4N$ HCl and then with boiling water till the washings were colourless. The precipitate, dried to constant weight, weighed 0·1207 g., which was 75·5 % of the theoretical yield. This proportion was found to be constant in a large number of experiments, including those with mixtures of glutamic acid, *iso*leucine and serine, showing only a variation from about 74 to 76 %, so that using this factor the method can be used for the determination of serine in solutions free from other hydroxyamino-acids with an experimental error of about 1·5 %.

In the case of mixtures of amino-acids the solution was first neutralized to litmus with alkali before the addition of chloramine-T.

Precipitability of serine by $Ca(OH)_2$ or $Ba(OH)_2$ and alcohol

0·20 g. of serine (containing 26·66 mg. N) was dissolved in 6 ml. of saturated $Ba(OH)_2$ solution in a centrifuge bottle and 60 ml. of alcohol were added slowly from a dropping funnel with vigorous shaking. The mixture was centrifuged for 5 min., the sticky precipitate washed twice with 6 ml. portions of alcohol, dissolved in 6 ml. of water and precipitated once again by gradual addition of 60 ml. of alcohol as before. On dissolving the precipitate and making up to volume (50 ml.) it was found to contain 63·78 % (17·03 mg.) of the N. The filtrate from the Ba-alcohol precipitate was freed from alcohol by evaporation *in vacuo* and contained 37·24 % (9·93 mg.) of the N.

Repetition of the experiment with $Ca(OH)_2$ and alcohol gave similar results.

SUMMARY

By digestion of 'paranuclein' from casein with trypsin an enzyme-resistant phosphopeptone of constant composition has been isolated in the form of its Ba salt.

The phosphopeptone has been shown to contain 10 amino-acid units, viz. 3 mol. of glutamic acid, 3 mol. of *iso*leucine and 4 mol. of serine. The absence of other hydroxy- or dicarboxylic-amino-acids has been demonstrated by indirect methods.

A method is described for the approximate estimation of serine in the absence of other hydroxyamino-acids.

It is shown that the presence of serine in high concentration interferes with the estimation of the dicarboxylic acids both by titration and by precipitation according to Foreman, in the former by the formation of secondary acidic decomposition products and in the latter on account of the partial precipitation of serine under the same conditions as the dicarboxylic acids.

REFERENCES

Abderhalden & Broich (1933). *Biochem. Z.* **262**, 321.
Cohn & Hendry (1930). *Organic Synth.* **10**, 16.
Daft & Coghill (1931). *J. biol. Chem.* **90**, 341.
Dakin (1916). *Biochem. J.* **10**, 319.
—— (1917). *Biochem. J.* **11**, 79.
—— (1918). *Biochem. J.* **12**, 290.
Damodaran (1931, 1). *Biochem. J.* **25**, 2123.
—— (1931, 2). *Biochem. J.* **25**, 190.

Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
Friedemann, Cotonio & Shaffer (1927). *J. biol. Chem.* **73**, 335.
Gulland & Morris (1934). *J. chem. Soc.* p. 1644.
Harington & Randall (1931). *Biochem. J.* **25**, 1916.
Harris (1929). *J. biol. Chem.* **84**, 298.
Jordan Lloyd & Shore (1938). Chemistry of the Proteins. London: J. and A. Churchill.
Kingston & Schryver (1924). *Biochem. J.* **18**, 1070.
Leib & Zacherl (1932). *Hoppe-Seyl. Z.* **211**, 211.
Lipmann (1933). *Biochem. Z.* **262**, 3.
Nef, Hedenberg & Glattfeld (1917). *J. Amer. chem. Soc.* **39**, 1638.
Posternak (1927). *C.R. Acad. Sci., Paris*, **184**, 306.
—— (1928). *C.R. Acad. Sci., Paris*, **186**, 1762.
Rapoport (1935). *Biochem. Z.* **289**, 406.
Rimington (1927, 1). *Biochem. J.* **21**, 1179.
—— (1927, 2). *Biochem. J.* **21**, 1187.
Simon & Neuberg (1931). *Biochem. Z.* **232**, 479.
Vickery (1922). *J. biol. Chem.* **53**, 495.

14. THE ACTION OF SULPHITES ON THE CYSTINE DISULPHIDE LINKAGES IN WOOL

2. THE INFLUENCE OF TEMPERATURE, TIME AND CONCENTRATION ON THE REACTION

By FRANK FLINTHAM ELSWORTH AND HENRY PHILLIPS

From the Wool Industries Research Association, Torridon, Headingley, Leeds, 6

(*Received 19 December 1940*)

A STUDY of the interaction of wool at room temperature with buffered sulphite solutions of different pH [Elsworth & Phillips, 1938, 1] showed that only a fraction of the total disulphide-S in the wool reacted at pH 5 according to equation (1) and much smaller amounts in solutions of lower or higher pH.

$$R_1.CH_2.S.S.CH_2.R_2+M_2SO_3\rightarrow R_1.CH_2.S.M=R_2.CH_2.S.SO_2.OM \qquad ......(1)$$

(R_1 and R_2, adjacent polypeptide chains).

The present investigation is concerned with the influence of other factors on this reaction, with the explanation of why it is apparently restricted to only a portion of the total disulphide-S of the wool, and also with the determination of the maximum proportion of the disulphide-S that will react in this manner.

EXPERIMENTAL

The influence of time on the reaction. Pieces of loosely woven, thin woollen fabric, prepared from 64–70's wool, were immersed for the times given in Table 1 in thirty times their weight of buffered $NaHSO_3$ solutions at room temperature containing respectively 1·0 and 2·5% SO_2; the pH was 4·5. The wool fabric was similar to that used in the previous investigation and the buffered $NaHSO_3$ solutions were also prepared in a similar manner. To avoid oxidation, the reactions were carried out in closed flasks. The bisulphited wools were rinsed in water to remove buffer salts and the bulk of the free bisulphite, dried at room temperature and then allowed to reach equilibrium with a controlled atmosphere of 21° and 70% R.H. Reaction (1) is reversible and bisulphited wool loses $NaHSO_3$ slowly when rinsed in water. For this reason, no attempt was made to wash bisulphited wools until they were free from $NaHSO_3$. The presence of free $NaHSO_3$ does not interfere with the methods of analysis employed [Elsworth & Phillips, 1938, 1, 2]. The conditioned wools were cut up into small pieces, their moisture contents determined by Barritt & King's method [1926] and their thiol-S contents, after hydrolysis for 4 hr. in $5N$ HCl (20 ml. per g.), by Shinohara's [1935, 1] modification of Folin & Marenzi's method [1929]. Two thiol groups arise in the hydrolysate of the wool for each disulphide group which reacts with the bisulphite, since the S-cysteinesulphonate group (equation (1)) is converted into a thiol group when the bisulphited wool is hydrolysed [Elsworth & Phillips, 1938, 1].

$$R_2.CH_2.S.SO_2.OM+HCl\rightarrow R_2.CH_2.SH+MCl+H_2SO_4. \qquad ......(2)$$

Table 1. *The influence of time of treatment on the reaction between wool and solutions of* $NaHSO_3$ *at* pH *4·5 containing respectively 1·0 and 2·5 %* SO_2

	Thiol-S in hydrolysate, % of dry wool	
Time of treatment hr.	(*a*) From solution containing 1 % SO_2	(*b*) From solution containing 2·5 % SO_2
0·25	0·40	—
0·50	0·44	0·52
1	0·50	0·58
2	0·53	0·64
4	0·55	0·66
7	0·61	0·68
24	0·72	0·73
48	0·69	0·85
96	—	0·83
120	0·64	—
168	—	0·82
288	0·84	—
336	—	0·94

The results (Table 1) show that the rate of reaction is high in the early stages, but then gradually decreases. In both solutions, the reaction proceeds very slowly after 0·7 % of disulphide-S (calculated on the dry wool) has reacted.

The influence of temperature on the reaction. Pieces of the woollen fabric, after previous wetting-out, were heated for 30 min. with forty times their weight of buffered $NaHSO_3$ solutions of *p*H 4·9 containing 1 % of SO_2. They were then rinsed and dried, as described above, before analysis. Besides the thiol-S, the total SO_2 contents of the wools were determined by distillation with dilute H_3PO_4 (1 ml. H_3PO_4, sp. gr. 1·75 in 500 ml.; initial *p*H 2·1) [Elsworth & Phillips, 1938, 2]. The rinsed wools could not be considered to be free from $NaHSO_3$, so that a small amount of the total SO_2 returned by this method was derived from this source. The bulk of the SO_2 arose from the decomposition of *S*-cysteine-sulphonate groups according to equation (3).

$$R_2.CH_2.S.SO_2.ONa \xrightarrow{H_3PO_4} R_2.CH_2.SOH + NaHSO_3. \qquad ......(3)$$

Table 2. *The influence of temperature on the reaction between wool and a solution of* $NaHSO_3$ *at* pH *4·9 containing 1 %* SO_2

Temp. ° C.	Total SO_2, % of dry wool	Thiol-S in hydrolysate, as % of dry wool
20	0·72	0·45
30	0·83	0·62
40	0·75	0·65
50	0·83	0·69
60	0·97	0·72
70	0·98	0·74
80	1·01	0·76
90	0·91	0·76
100	0·75	0·66

From Table 2 it will be seen that increase in temperature up to 60° assists the reaction. At higher temperatures the wool was weakened, and at 100° it is probable that other chemical changes occurred in the wool besides the bisulphite reaction and caused the observed decrease in the thiol-S in the hydrolysate. Theoretically, since the weight of 2S atoms is the same as that of the SO_2 molecule, the weights of SO_2 obtained from these bisulphited wools should have equalled the weights of thiol-S found in the hydrolysates. In every wool, the total SO_2 is greater than the thiol-S because of the incomplete removal of the uncombined $NaHSO_3$ before analysis.

The influence of the concentration of NaHSO₃. Pieces of the woollen fabric (10 g.) were immersed for 24 hr. at room temperature in 300 ml. of buffered $NaHSO_3$ solutions (*p*H 5·3–5·7) containing 0·1–5·0 % SO_2. The subsequent treatment of the wools before the determination of the thiol-S contents of their hydrolysates was as previously described. In Fig. 1 the thiol-S contents of the hydrolysates of the bisulphited wools are plotted against the concentrations of the solutions in which the wools were treated. It will be seen that the extent of the reaction increases at first, but reaches a maximum when the solution contains 8 % SO_2 (13 % $NaHSO_3$).

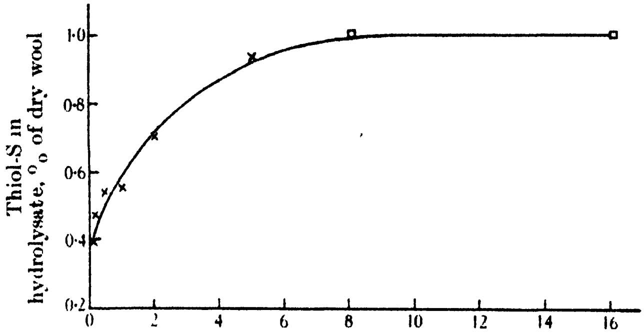


Fig. 1. SO_2, % in solution, *p*H 5·3–5·7. ×—× Commercial fabric. □—□ Australian 64's.

These observations were extended using a virgin wool which contained very nearly the same percentage of disulphide-S as the wool of the commercial fabric. This virgin wool was prepared from a 64's Australian merino fleece by extracting a picked portion three times with benzene at 40° and then removing dirt, vegetable matter and discoloured fibres by hand. The remaining wool, after it had been extracted three times with benzene and twelve times with water, was finally washed in an 0·05 % solution of saponin followed by twelve rinses in water. After drying at room temperature and allowing to come to equilibrium with a controlled atmosphere of 21° and 70 % R.H., the clean wool contained 3·42 % total S and 3·09 % disulphide-S (calculated on its anhydrous weight). Samples of this wool were immersed for 24 hr. in fifty times their weight of solutions of $NaHSO_3$ of *p*H 5·7 containing respectively 8 and 16 % SO_2. The thiol-S values found in the hydrolysates of these bisulphited wools are plotted in Fig. 1, and it will be seen that they fall on the curve drawn through the points given by the wool of the commercial fabric. This wool contained 3·60 % S and 2·99 % disulphide-S. The curve in Fig. 1 possibly gives an exaggerated idea of the regularity of the reaction. A wider variety of different wools needs to be examined before the precise fraction of the disulphide-S that reacts with $NaHSO_3$ can be stated definitely.

After both these wools had been bisulphited, their original disulphide-S could be almost entirely accounted for either as thiol-S or as disulphide-S: hence during bisulphiting at room temperatures no conversion of disulphide-S into non-disulphide-S[1] took place. For example, after exposure for 24 hr. to the

---

[1] The term non-disulphide-S is used throughout this paper to describe S not returned either as thiol-S or as disulphide-S by Shinohara's method or as methionine-S by Baernstein's method.

$NaHSO_3$ solution containing 8 % SO_2, the virgin wool when hydrolysed gave 1·01 % *thiol-S* and 1·93 % disulphide-S, thus accounting for 2·94 % S or nearly all the total of 3·09 % S originally present as disulphide. This conclusion is of interest in the light of analytical results obtained when more drastic conditions for bisulphiting wool were used. Samples of the virgin wool were immersed in $NaHSO_3$ solutions at *p*H 5·7 containing 6·2 % SO_2 which were maintained at 40°. After 5¾ and also after 19 days' exposure to this solution, the wools gave 1·18 and 1·12 % thiol-S respectively. These percentages are somewhat higher than those produced by the treatment of wool with $NaHSO_3$ at room temperature. It is doubtful, however, whether this increase in thiol-S is due to an increase in the reaction between the wool and $NaHSO_3$ according to equation (1). At the higher temperature, other reactions occur which may increase the amount of thiol-S produced.

The occurrence of such reactions is shown by the conversion of disulphide-S into non-disulphide-S which took place. Thus the original wool contained 3·09 % disulphide-S, but after 5¾ days' treatment in $NaHSO_3$ at 40° only 2·76 %, and after 19 days only 2·49% of S in the wool could be accounted for either as disulphide- or thiol-S after hydrolysis.

The treatment of wool with concentrated solutions of $NaHSO_3$ (containing 8 % or more of SO_2) at room temperatures thus appears to lead to a straightforward reaction in which about one-third of the disulphide-S is converted into thiol and *S*-cysteinesulphonate groups. If the temperature of the $NaHSO_3$ solution is raised, even to 40°, other changes take place slowly and disulphide-S is converted into S which is neither thiol-S nor S present in *S*-cysteinesulphonate groups.

The reaction between $NaHSO_3$ and the cortical cells of wool. Although wool is attacked very slowly by a solution of papain, it has been found [Phillips, Middlebrook & W.I.R.A., 1939] that when $NaHSO_3$ is added to the papain solution, the wool is rapidly reduced to a mass of cortical cells, the intercellular protein and scales being digested. Cortical cells produced by this method were used to obtain evidence that they react with $NaHSO_3$. A 64's merino wool, which had been scoured commercially, was extracted with alcohol and then rinsed repeatedly in water. A sample of the wool was immersed for 30 min. in forty times its weight of solution of $NaHSO_3$ at *p*H 5·7 containing 2·0 % SO_2. The wool and solution were maintained at 65°. The bisulphited wool was rinsed with water and then analysed for thiol-S, free and combined SO_2 and for sulphate-S. A further sample of the wool (40 g.) was digested at 65° in 1 l. of 0·05 % papain solution containing 1 % $NaHSO_3$. After 17 hr., the digest was centrifuged and the insoluble material digested for a further 17 hr. with a fresh enzyme solution. The cortical cells that remained were centrifuged repeatedly with water until the supernatant liquor no longer gave a turbidity on the addition of H_2O_2 and $BaCl_2$. After drying *in vacuo* over P_2O_5 to constant weight, they were analysed for total S and thiol-S. They did not contain either free or combined SO_2, indicating that the bisulphite which they acquired in the enzyme solution had been removed completely. A sample of the cortical cells was then bisulphited under exactly the same conditions as the wool. The bisulphited cells were removed by centrifuging the solution and were washed with water. After drying *in vacuo* over P_2O_5, they were analysed again, the results of this analysis and those of the previous analyses being given in Table 3 as a percentage of the dry wool and cells. The total and 'free' SO_2 were determined by Elsworth & Phillips' methods [1938, 2].

Table 3. *Analysis of wool and wool cortical cells before and after treatment with $NaHSO_3$*

	Total S %	Thiol-S in hydrolysate %	Free SO_2 %	Combined SO_2 %	Sulphate-S %
Wool	3·64	0·10	—	—	0·08
Wool after bisulphiting	4·21	0·76	0·0	0·74	0·28
Cortical cells	4·12	0·17	0·0	0·0	—
Cortical cells after bisulphiting	5·09	0·75	0·23	0·76	0·66

The analyses of thiol-S, after hydrolysis, and the combined SO_2, show that very nearly the same percentages of the disulphide-S of the wool fibre and of the cortical cells react with $NaHSO_3$. This agreement may be accidental, since changes in the disulphide-S of the wool and cells, leading to its conversion into unreactive non-disulphide-S, may have occurred at unequal rates when the wool and cells were warmed with $NaHSO_3$ at 65° for 30 min. In addition, during their isolation the cortical cells were exposed for two periods of 17 hr. to 1 % $NaHSO_3$ at 65°.

The data of Table 3 provide some evidence as to the S content of the cortical cells and how this compares with that of the whole wool fibre. The S content of the cells can be calculated from the analyses after sulphiting, since these include a determination of sulphate-S by Mease's [1934] method. Allowing for sulphate-S, and S present as free and combined SO_2, the S content of the cells is 3·94 % calculated on their anhydrous weight. This exceeds the organic S, namely 3·56 %, of the wool from which they were isolated. Other evidence is available showing that the S content of the cellular components of the wool fibre exceeds that of the intercellular material. Stakheyowa-Kaverznewa & Cavrilov [1937] obtained an extract by the pancreatin-enterokinase digestion of wool which contained less S than the residual cells. They concluded that wool contained some 10 % of an intercellular phase of low S content. Speakman & McMahon [1938] digested the root portions of non-medullated Australian wool fibres with trypsin (thus avoiding the presence of light-damaged fibres with might be preferentially attacked by the enzyme) and found that the residual wool had a higher S content than the original wool. Their figures agreed with the presence of some 9 % of S-free protein in wool.

The reaction between alkali-degraded wool and $NaHSO_3$. On the hypothesis that $NaHSO_3$ reacts with a labile fraction of the disulphide-S of wool, it was thought possible that such S could be removed preferentially by alkali and the wool would not then react to the same degree, if at all, with $NaHSO_3$. The wool for these experiments was prepared by immersing pieces of the wool fabric for 30 min. in boiling buffer solutions of *p*H 8–10. The buffer solutions were borax-boric acid buffers and were 0·2 *M* in borate ion, except those of *p*H 9·6 and 10·0, which were 0·15 and 0·12 *M* respectively. The total S contents of the untreated and treated wools, after these had been rinsed thoroughly with water, were determined by the Denis-Benedict method as applied to wool by Barritt [1934]. The disulphide-S remaining intact in the wools was determined by Shinohara's method [1935, 2]. The pieces of fabric (about 10 g.) were then immersed for 20 hr. in 2800 ml. of a solution of $NaHSO_3$ at *p*H 5·0 containing 1·5 % SO_2. After passing through a wringer, the wools were immersed in water for 1 hr., allowed to dry in air and then to come to equilibrium with a controlled atmosphere of 21° and 70 % R.H. They were then analysed for thiol-S, and free and total SO_2, from which the combined SO_2 content was calculated.

Table 4. *Analyses of alkali-treated wools before and after reaction with* $NaHSO_3$

		After alkaline treatment followed by reaction with $NaHSO_3$			
(i) *p*H of buffer with which wool was boiled	*(ii)* Total-*S*, % after treatment	*(iii)* Total SO_2 %	*(iv)* Free SO_2 %	(v) Combined SO_2 %	(vi) Thiol-*S* in hydrolysate %
(Untreated)	3·60	0·79	0·23	0·56	0·61
10·0	2·52	0·45	0·21	0·24	0·22
9·6	2·90	0·47	0·21	0·26	0·28
9·2	3·05	0·53	0·21	0·32	0·37
8·9	3·29	0·55	0·09	0·46	0·45
8·5	3·56	0·64	0·17	0·46	0·47
8·1	3·51	0·69	0·16	0·53	0·53

An important deduction from these results (Table 4) is that the amount of reaction that occurs is proportional to the disulphide-S contents of the wools as recorded in Table 5. Thus the thiol-S values in the hydrolysates of the wools (Table 4, col. (vi)) are approximately one-fifth of the disulphide-S contents of the wools (Table 5, col. (ii)), suggesting that no matter how small the disulphide-S becomes, one-fifth will react with $NaHSO_3$ under the conditions of these experiments. The residual disulphide-S of alkali-treated wools thus appears to react as freely with $NaHSO_3$ as the disulphide-S of untreated wool.

Another significant feature of these results is that for each thiol group in the alkali-treated and bisulphited wools there is one *S*-cysteinesulphonate group. This is indicated by the very good agreement between the figures for the combined SO_2 and the thiol-S in the hydrolysates (Table 4, cols. (v) and (vi)). When the extent and possible nature of the chemical alteration produced in the wool by alkalis is taken into consideration, this agreement is remarkable. For example, most of the chemical changes which it has been suggested that wool undergoes when treated with alkalis are assumed to give rise to free thiol groups. Speakman [1933] has suggested that mild treatment with alkali may cause hydrolysis of the disulphide linkage, followed, under certain conditions, by condensation between the sulphenic acid groups and adjacent amino-groups.

$$.CH_2.S.S.CH_2.\rightarrow.CH_2.SH+.CH_2.SOH, \qquad \ldots\ldots(4)$$

$$.CH_2.SOH+H_2N.\rightarrow.CH_2.SNH.+H_2O, \qquad \ldots\ldots(5)$$

$$.CH_2.SOH\rightarrow.CHO+H_2S. \qquad \ldots\ldots(6)$$

These changes ((4) and (5)) occur without loss of S. When S is lost (as in (6)) Schöberl [1933] has suggested that the sulphenic acid groups decompose giving aldehyde groups and H_2S. Race *et al.* [1938] have obtained evidence that a decomposition similar to that postulated by Schöberl occurs in the tips of wool fibres when they are exposed to light and moisture during growth. They found that such light-damaged wool contained aldehyde groups, but less S than the corresponding root wool. Speakman & McMahon [1938] titrated wool from the tips and from the root ends of a fleece and from the differences between the titration curves concluded that the tips of the fibres contained appreciable quantities of thiol groups, whereas the root ends contained only traces. The evidence for the presence of thiol groups in wool which has undergone chemical change in aqueous solutions is not so strong. Crowder & Harris [1936] caused wool to lose half its S, in accordance with the Schöberl reaction, by exposing it to a current of aqueous NaOH. They were unable to find any cysteine in the hydrolysate of the wool and assumed that this was due to the formation of thioacetals during the hydrolysis.

Assuming that disulphide-S is destroyed by the reactions suggested by Speakman and Schöberl, an estimate of the amounts of thiol-S which were formed in the alkali-treated wools can be made from the analyses recorded in Tables 4 and 5. In these analyses, the difference between the values of the disulphide-S and total S minus the value of the methionine-S (found to be 0·10 % using Baernstein's method [1932] for the determination of methionine) gives the amount of S present in the wools as non-disulphide-S. In the untreated wool, the non-disulphide-S amounts to 0·51 % on the weight of the anhydrous wool, and is considered to have arisen without loss of S. This may not be true, since the wool may have lost S by exposure to air, moisture and light during growth, but the amount is unlikely to be large, since the total S of the wool is 3·60 %, a reasonably high value. The total non-disulphide-S in the alkali-treated wools is subdivided by assuming that for each atom of S lost by the wool, one atom of non-disulphide-S is produced. The non-disulphide-S produced with loss of S is then subtracted and figures for the non-disulphide-S produced without loss of S are obtained.

Table 5. *The modification of the disulphide-S of wool when it is treated with boiling buffer solutions*

*p*H of buffer with which wool was boiled	Disulphide-S, % after treatment	Non-disulphide-S, % produced without loss of S	Non-disulphide-S % produced with loss of S
–	2·99	0·51	—
8·1	2·59	0·73	0·09
8·5	2·36	1·06	0·04
8·9	1·87	1·01	0·31
9·2	1·57	0·83	0·55
9·6	1·31	0·79	0·70
10·0	1·04	0·30	1·08

It will be seen (Table 5) that when wool is treated with boiling mildly alkaline solutions relatively large amounts of non-disulphide-S are produced without loss of S: in the more strongly alkaline solutions, non-disulphide-S is produced with loss of S. The significance of these changes will be more fully discussed in a subsequent paper. Here we need only point out that if the non-disulphide-S was produced according to the Speakman and Schöberl reactions, part of it should have been found as thiol-S. For example, the hydrolysate of the wool boiled with the buffer of *p*H 9·2 should have contained 0·42 % thiol-S arising by the Speakman reaction and 0·55 % thiol-S by the Schöberl reaction, a total of 0·97 %, calculated on the weight of wool hydrolysed. Experimentally none was found. On the other hand, when this alkali-treated wool was bisulphited, and determinations of the combined SO_2 indicated that theoretically the hydrolysed wool should contain 0·32 % thiol-S, 0·37 % was actually found.

In some instances, it is not possible to attribute the failure to find the thiol-S to the presence of aldehydes in the hydrolysate of the wool. For example, the non-disulphide-S in the wool increased from 0·51 to 1·06 % when it was boiled in buffer of *p*H 8·5, but the wool lost only 0·04 % S. If the additional non-disulphide-S arose mainly by the Speakman reaction, 0·25 % thiol-S was produced without the simultaneous production of aldehyde groups. In spite of this, no increase in the thiol-S of the hydrolysed wool was observed. It appears probable therefore that if disulphide cross-linkages are destroyed according to the Speakman and Schöberl reactions, the thiol-S produced must become linked to other atoms in the keratin molecule.

DISCUSSION

The present investigation has shown that whilst the reaction between wool and $NaHSO_3$ proceeds quickly at first, there is a point at which it is difficult to cause further reaction to take place. This is brought out most clearly in Fig. 1, which shows the effect of increasing the concentration of the $NaHSO_3$. In solutions containing up to 8 % SO_2 at room temperatures about one-third of the disulphide-S of the wool reacts and a twofold increase in concentration produces no further reaction. At higher temperatures, although increased amounts of disulphide-S disappear from the wool, there is evidence that the wool has not combined with equivalent amounts of $NaHSO_3$.

It is, however, important to emphasize that all the bisulphited wools mentioned in this paper were analysed after they had been rinsed with water. It is known [Elsworth & Phillips, 1938, 1] that $NaHSO_3$ combined with wool is not readily removed by water, but further work is necessary to prove whether all the $NaHSO_3$ removed when bisulphited wool is rinsed with water is free $NaHSO_3$ and not $NaHSO_3$ less firmly combined with some additional fraction of the disulphide-S.

Nevertheless sufficient evidence has been obtained to suggest that the reactivity of one-third of the disulphide-S of wool towards $NaHSO_3$ differs from that of the remaining two-thirds. This does not necessarily mean that one-third of the disulphide-S of wool is more reactive than the remaining two-thirds. If this were so, we should expect to be able to remove the more reactive fraction by means of alkali, and obtain a wool which would not react with $NaHSO_3$. Experimentally it is found, however, that the residual disulphide-S in alkali-treated wools is as reactive towards $NaHSO_3$ as the disulphide-S of untreated wool.

A more likely hypothesis appears to be that whilst all the disulphide linkages of wool are equally reactive towards $NaHSO_3$, after this reagent has reacted with and broken some of these linkages, stresses on the remaining linkages are dissipated and their reactivity is altered. In agreement with this suggestion, it is known that the physical properties of hair are profoundly changed by boiling it for 30 min. with a 5 % solution of $NaHSO_3$. Speakman [1936] found that such treatment enabled human hair to supercontract 30 % in length when it dried. Assuming that human hair reacts with $NaHSO_3$ in the same manner as wool, it would appear that this supercontraction becomes possible because about one-third of the disulphide linkages in the hair have been broken. These linkages may therefore be responsible for maintaining the keratin in the α-configuration: when they are broken by $NaHSO_3$, the keratin molecule can assume a more folded and shorter configuration [Astbury & Woods, 1933].

Unstretched human hair that has been boiled with a 2 % solution of borax (*p*H 9·2) does not contract in length when boiled with 5 % $NaHSO_3$. If it is first boiled with dilute HCl and then with 5 % $NaHSO_3$, it contracts 15 % in length [Speakman & Stoves, 1937]. These authors assume that for each disulphide linkage destroyed by the borax another linkage is produced. This may either be a —S—NH— linkage arising without loss of S, or, when S is lost, a —CH=NH— linkage arising, as suggested by Phillips [1936], by condensation between the aldehyde group and an adjacent amino-group. Boiling the hair in dilute HCl may break the —CH=N— cross-linkages, so that after additional linkages have been broken by $NaHSO_3$, the total number of linkages broken is then sufficient to enable some supercontraction to take place.

SUMMARY

1. The disulphide-S of wool reacts with $NaHSO_3$ and thiol and *S*-cysteinesulphonate groups are formed which are not readily removed by rinsing the bisulphited wool with water. By increasing the temperature, time of reaction and concentration of $NaHSO_3$ not more than one-third of the total disulphide-S of the wool can be caused to react in this manner.
2. The cortical cells, isolated by digestion of wool with papain in the presence of $NaHSO_3$, contain more organic S than the fibre as a whole. The keratin of the cells reacts with $NaHSO_3$ to give thiol and *S*-cysteinesulphonate groups.
3. The residual disulphide-S in wool that has been boiled with alkaline solutions of *p*H 8 and higher reacts with $NaHSO_3$ in the same proportion as the disulphide-S of untreated wool.

The authors are indebted to Mr W. R. Cuthbertson for the experiments with the cortical cells and to Mr W. R. Middlebrook for the experiments with virgin wool. Thanks are also due to the Council of the Wool Industries Research Association for permission to publish this paper.

REFERENCES

Astbury & Woods (1933). *Philos. Trans.* A, **232**, 333.
Baernstein (1932). *J. biol. Chem.* **97**, 663.
Barritt (1934). *J. Soc. chem. Ind., Lond.*, **53**, 291 T.
—— & King (1926). *J. Text. Inst., Manchr.*, **17**, 386.
Crowder & Harris (1936). *J. Res. Nat. Bur. Stand.* **16**, 475.
Elsworth & Phillips (1938, 1). *Biochem. J.* **32**, 837.
—— —— (1938, 2). *J. Text. Inst., Manchr.*, **29**, 219 T.
Folin & Maronzi (1929). *J. biol. Chem.* **83**, 103.
Mease (1934). *J. Res. Nat. Bur. Stand.* **13**, 617.
Phillips (1936). *Nature, Lond.*, **138**, 121.
—— Middlebrook & W. I. R. A. (1939). B.P. 513,919.
Race, Rowe, Speakman & Vickerstaff (1938). *J. Soc. Dy. Col., Bradford*, **54**, 141.
Schoberl (1933). *Liebigs Ann.* **507**, 111.
Shinohara (1935, 1). *J. biol. Chem.* **109**, 665.
—— (1935, 2). *J. biol. Chem.* **112**, 671.
Speakman (1933). *Nature, Lond.*, **132**, 930.
—— (1936). *J. Soc. Dy. Col., Bradford*, **52**, 335.
—— & McMahon (1938). *Nature. Lond.*, **141**, 118.
—— & Stoves (1937). *J. Soc. Dy. Col., Bradford*, **53**, 236.
Stakheyewa-Kaverznewa & Cavrilov (1937). *Bull. Soc. Chem. (Mémoires)*, **4**, 647.

15. A STUDY OF THE ISOTOPIC CONSTITUTION OF POTASSIUM IN VARIOUS RAT TISSUES

By ARTHUR LASNITZKI

Cancer Research Department, University of Manchester;
Department of Physiology, University of Birmingham

AND A. KEITH BREWER

Bureau of Plant Industry, U.S. Department of Agriculture, Washington, D.C.

(*Received 10 December 1940*)

PRELIMINARY investigations [Brewer, 1936; 1937; Lasnitzki & Brewer, 1938] have disclosed the somewhat surprising fact that the abundance ratio of the isotopes of potassium is not a constant figure for all organic tissues. Since the observed isotope effect is necessarily associated with processes involved in K metabolism, it was considered advisable to make a comprehensive study of the isotope ratio in various animal tissues in hope that it would contribute to our knowledge of the mechanism of K assimilation. Various tissues from the rat, including red blood corpuscles and blood plasma, have been chosen for investigation.

Potassium is known to have three natural isotopes, designated as: ${}^{39}_{19}K$, ${}^{40}_{19}K$ and ${}^{41}_{19}K$, the indices '39', '40' and '41' indicating the mass number and the common index '19' the nuclear charge. The 39-isotope represents the bulk of the element, the 41-isotope a relatively small fraction, while the 40-isotope (which is radioactive) exists only in a very minute quantity. The present study is concerned only with the 39- and 41-isotopes.

Methods

Preparation of ashes. Adult or preadult rats, taken from mixed strains, were studied first. The animals were anaesthetized with chloroform, and, after opening the chest, blood was drawn from the heart, usually by puncture of the left ventricle. The blood was cooled on ice, mixed with a small amount of 5% sodium citrate solution to prevent clotting, and then centrifuged at high speed. The citrate plasma was separated, centrifuged again and filtered through an ash-free filter. The red blood corpuscles were freed from residual plasma and white blood cells by washing them several times with 0·9% NaCl. Plasma and blood corpuscles were then dried at about 100°, part in glass and part in silica vessels.

The solid tissues were taken immediately after the recovery of the blood. The soft organs were freed as far as possible from adhering connective tissue, etc., and cut into small pieces; these were rapidly rinsed in distilled water and dried on Petri dishes at the above temperature. The bones were treated similarly. By the cardiac puncture and subsequent section of the venae cavae the blood content of the tissues was lowered appreciably. When no heart puncture was made, the animal was bled in a similar way after being chloroformed.

Some solid tissues were taken also from new-born rats. The animals were killed by decapitation and freed as far as possible from blood. The tissues were treated as above.

After drying, the corresponding material obtained from different animals was mixed and then ashed, wholly or in part. The ashing was carried out in a Pt dish, which had been previously cleansed and ignited to bright redness in order to remove any K possibly absorbed by the metal. The number of animals employed in the preparation of one ash sample varied considerably. Among the adult and preadult animals an equal number of males and females was as a rule utilized.

Mass-spectrographic measurement. The ash prepared in this way was used, without further treatment, for the determination of the isotopic ratio ${}^{39}_{19}\mathrm{K}/{}^{41}_{19}\mathrm{K}$ by means of a mass-spectrograph. The special type of apparatus employed was previously described in detail [Brewer, 1938]. The essential parts are shown schematically in Fig. 1. A Pt disk, 1·5 mm. in diameter, was the source of the K atoms. The disk was spot-welded to a tungsten filament which was heated by a heavy duty storage cell. Before coating, the disk was ignited at white heat until all residual K was removed. A small quantity of ash was then placed on the disk and heated in a vacuum to about 700° for 30 min. During this heating process the Pt absorbed sufficient K to become an excellent emitter of K ions after the ash had been carefully scraped away. The impregnated disk was mounted at a distance of 5–8 mm. directly above the entrance slit to the analysing chamber. This arrangement ensured that all the potassium ions leaving the disk and impinging on the slit had attained the same velocity. Between the disk and the slit a potential drop of 540 v. was maintained, the disk being positive. By surrounding the disk with a washer type shield, maintained positive to the disk by about 60 v., it was possible to bring all the emitted ions to a focus on the slit, and by a special arrangement (not illustrated) the slit could be placed directly in the centre of the path of the focussed ion beam. When the disk was brought to a temperature of about 600°, the positive ion current from the disk to the slit was of the order of 10^{-9} to 10^{-10} amp.

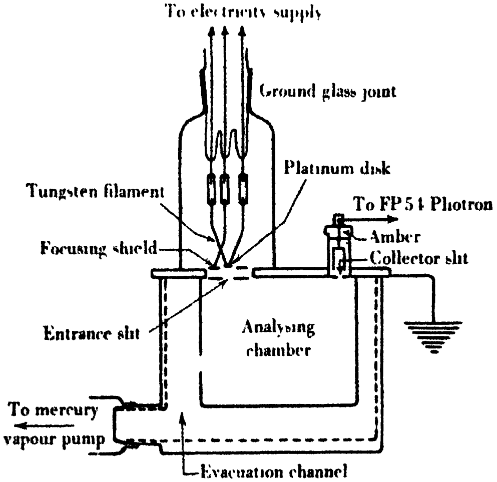


Fig. 1. Diagram of mass-spectrograph.

The ion current entering the analysing chamber was resolved by means of a magnetic field produced by an electromagnet with poles directed against the side walls of the chamber, which were made of Swedish iron. By adjusting the current to the magnet it was possible to bring all the ions of the same mass number to a focus at the collector slit. Since the latter was wide with respect to the entrance slit, the entire resolved ion beam was caught by the collector. The intensity of each resolved ion current was recorded by means of an FP54 Pliotron. The ratio of the galvanometer deflexions at the 39 and 41 mass positions was equal to the isotopic ratio $^{39}_{19}$K/$^{41}_{19}$K, since under the given conditions the deflexions were linear over a large scale. From the isotopic ratio the percentage of the heavy isotope $^{41}_{19}$K was calculated, neglecting the very minute amount of $^{40}_{19}$K.

The most common error occurring in these measurements was associated with the 'background' resulting from the scattering in the resolved ion beams. Since accurate measurements could not be performed in the presence of background, all measurements were excluded in which the galvanometer deflexion at the 40 mass position exceeded 0·1 mm. Another error might have arisen from the platinum disk failing to take up the two isotopes exactly in the same ratio in which they were present in the ash; careful tests showed no evidence for such a possibility. In practice about thirty estimations of the isotopic ratio were always made in succession on each ash sample. While individual estimations showed some variation, the average of any ten estimations usually agreed to within ±0·01, corresponding to $\sim \pm 0{\cdot}005\,\%$ $^{41}_{19}$K.

Results

Ordinary commercial KCl (A.R.) served as a normal standard for the comparison with the K contained in tissues. For the mass-spectrographic analysis the chloride was converted into the perchlorate, which could be used in the same manner as the ash. The isotopic ratio $^{39}_{19}$K/$^{41}_{19}$K determined with this preparation was 14·20, the $^{41}_{19}$K content being 6·579 %. Results obtained with ashes from various tissues are shown in Tables 1–5, each corresponding to one particular group of tissues.

In Table 1 the results from liver, kidney, lung and salivary gland are given. It will be seen that the isotopic ratios are, in the majority of cases, very close to that of mineral K. On the other hand, there are three values—one each with liver, kidney and salivary gland—which are very slightly increased, more than can be accounted for by the experimental error. The content of $^{41}_{19}$K was thus decreased (by 0·5–0·7 %). Ash sample no. 4 is of special interest. The livers used for the preparation of this sample were perfused *in situ* through the portal vein by K-free Ringer's solution with an increased content of Ca. This perfusion

Table 1. *Analysis of K in liver, kidney, lung and salivary gland*

Sample no.	Tissue	No. of animals used	Average animal wt. in g. (approx.)	Isotopic ratio $^{39}_{19}$K/$^{41}_{19}$K	% of $^{41}_{19}$K
1	Liver	8	210	14·20	6·579
2	,,	4	220	14·27	6·549
3	,,	4	150	14·20	6·579
4	Liver (perfused)	2	235	14·23	6·566
5	Kidney	12	210	14·28	6·545
6	,,	6	215	14·22	6·570
7	,,	4	150	14·20	6·579
8	Lung	12	210	14·24	6·562
9	,,	4	150	14·20	6·579
10	Salivary gl.	16	215	14·31	6·532
11	,, ,,	10	210	14·21	6·575
12.	,, ,,	6	145	14·20	6·579

removed almost completely the blood present in the tissue. The effect on the isotopic ratio was, however, negligible or zero.

Table 2 contains the results from skeletal muscle, heart muscle and brain. The values of the isotopic ratio do not show any noticeable deviation from that of mineral K, except one sample from skeletal muscle. Here again a minute increase of the isotopic ratio was observed, and a corresponding decrease of the $^{41}_{19}K$ content (by about 0·4 %).[1]

The tissues dealt with in Table 3, i.e. spleen, lymph glands, testis, and bone including marrow, are distinguished from the above tissues by the greater rate of proliferation of their cellular elements. It will be seen that with ashes from spleen, lymph glands and testis no noticeable deviation of the isotopic ratio was found. A marked difference, however, was obtained with ashes from bone including marrow. In each of the five samples analysed the isotopic ratio was clearly decreased, and the content of $^{41}_{19}K$ thus increased. The value of this increase showed some variation. In three of the samples it was 1·5–1·7 %, in one sample about 2·2 %, and in one sample about 2·8 %. The average was 1·9 %.

Table 2. *Analysis of K in skeletal muscle, heart muscle and brain (cortex and medulla)*

Sample no.	Tissue	No. of animals used	Average animal wt. in g. (approx.)	Isotopic ratio $^{39}_{19}K/^{41}_{19}K$	% of $^{41}_{19}K$
1	Skeletal muscle	8	210	14·26	6·553
2	„ „	4	220	14·20	6·579
3	„ „	4	150	14·20	6·579
4	Heart muscle	12	210	14·20	6·579
5	Brain	12	210	14·22	6·570
6	„	4	155	14·19	6·583

Table 3. *Analysis of K in spleen, lymph glands, testis and bone including marrow*

Sample no.	Tissue	No. of animals used	Average animal wt. in g. (approx.)	Isotopic ratio $^{39}_{19}K/^{41}_{19}K$	% of $^{41}_{19}K$
1	Spleen	12	210	14·22	6·570
2	Lymph glands	16	225	14·20	6·579
3	Testis	12	250	14·20	6·579
4	Bone including marrow*	8	220	13·95	6·689
5	Do.*	2§	250	13·97	6·680
6	Do.*	2‖	195	13·97	6·680
7	Do.†	4	150	13·79	6·761
8	Do.‡	1§	225	13·88	6·721

* Diaphyses of humerus, femur and tibia of both sides.
† Diaphyses of femurs only.
‡ Diaphyses of left bones only.
§ Males only. ‖ Females only.

---

[1] Another analysis not included in the table was carried out with a sample of $KClO_4$, prepared by $HClO_4$ precipitation from an extract of rabbit muscle. No deviation of the isotopic ratio was found. This result agreed with previous findings by Lasnitzki & Oeser [1937] concerning the radioactivity of K in rabbit muscle, a similar preparation (after its conversion into the chloride) having been used.

Further, it was found that ash prepared from a humerus, femur and tibia, after complete removal of the marrow from the cavity, showed an isotopic ratio of 14·03, corresponding to a $^{41}_{19}K$ content of 6·653 %. The bones were taken from the right side of the same animal from which the corresponding bones of the left side constituted ash sample no. 8. Thus K contained in the bone substance appears to show a smaller deviation of its isotopic ratio than K derived from both bone substance and marrow. K from marrow alone would show, therefore, rather a greater deviation of its isotopic ratio than that which was actually found.

The conclusion that the K present in bone marrow of the rat has a higher content of $^{41}_{19}K$ than ordinary mineral K agrees with similar findings previously obtained with marrow from horse and beef [Brewer, 1937].

The results obtained with red blood corpuscles and blood plasma are shown in Table 4. It is evident that the isotopic ratio of K in the ash of red blood corpuscles was not different from normal. On the other hand, a marked decrease was observed in the ash of blood plasma. The deviation was clearly indicated with each of the five plasma samples, yet with two of them more pronouncedly than with the others. The corresponding increases in the content of $^{41}_{19}K$ were 3·0–3·2 and 1·8–2·3 % respectively; the average increase was 2·5 %.

Table 4. *Analysis of K in red blood corpuscles and blood plasma*

Sample no.	Source	No. of animals used	Average animal wt. in g. (approx.)	Isotopic ratio $^{39}_{19}K/^{41}_{19}K$	% of $^{41}_{19}K$
1	Red blood corpuscles	12	220	14·20	6·579
2	,, ,, ,,	6	220	14·19	6·583
3	,, ,, ,,	4	155	14·20	6·579
4	Blood plasma	12	220	13·74	6·784
5	,, ,,	6	220	13·92	6·702
6	,, ,,	6	215	13·72	6·793
7	,, ,,	2	200	13·87	6·725
8	,, ,	4	155	13·89	6 716

A special test was carried out to determine whether the very high Na content of the plasma ash, due to the mixture of blood and sodium citrate solution, might have interfered with the mass-spectrographic measurement; although there was no theoretical reason for such a possibility. Dry liver and muscle tissue were thoroughly mixed with sodium citrate in a ratio approximating plasma. The ashes of these mixtures and those of the plain dry tissues were compared respectively, but no difference in the isotopic ratio was found. Thus the high preponderance of Na in the plasma ash did not interfere with the measurements. (The same can be said with regard to Ca in bone ash, since the addition of a large amount of calcium phosphate to dry tissue did not affect the isotopic ratio.)

In Table 5 are given the results for liver, kidney and skeletal muscle, taken from new-born animals. While K contained in the ash of liver and muscle tissue showed about the same isotopic ratio as mineral K, that present in kidney tissue showed a minute increase of this value, and thus a slight decrease in the $^{41}_{19}K$ content (by about 0·7 %). This deviation is comparable with that obtained with one of the ash samples from adult kidney, but does not agree with the absence of any noticeable deviation in the two other samples. On the whole, no difference seems to exist in the isotopic ratios of K animals in these three tissues in relation to the age of the animal.

Table 5. *Analysis of K in liver, kidney and skeletal muscle of new-born animals*

Sample no.	Tissue	No. of animals used	Average animal wt. in g. (approx.)	Isotopic ratio $^{39}_{19}K/^{41}_{19}K$	% of $^{41}_{19}K$
1	Liver	111	5·5	14·19	6·583
2	Kidney	127	5·5	14·30	6·536
3	Skeletal muscle	7	5·0	14·21	6·575

A representation of the whole of our results is shown in Fig. 2. The height of each column indicates the % of $^{41}_{19}K$ found in K contained in one particular ash sample, while the dotted line indicates the % of $^{41}_{19}K$ in mineral K. The columns are interrupted between 0·2 and 6·4 % $^{41}_{19}K$.

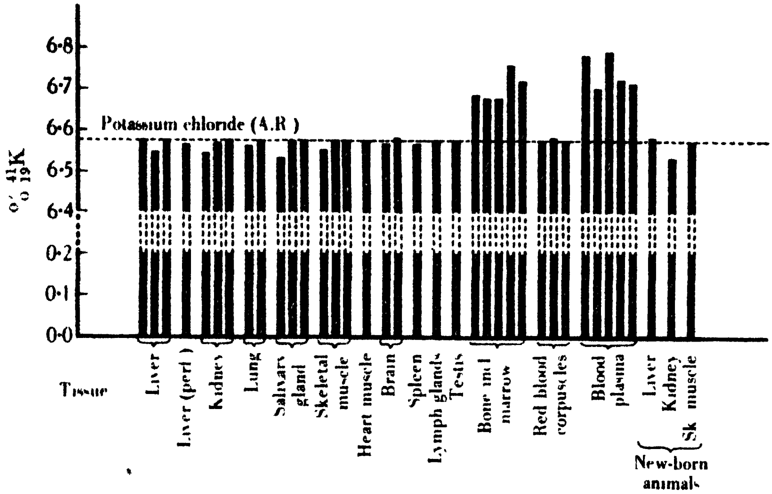


Fig. 2. Content of $^{41}_{19}K$ in potassium present in various rat tissues.

DISCUSSION

Considering the results as a whole, it appears that a constant deviation from the isotopic ratio of mineral K was observed only for K present in bone marrow and blood plasma. In both cases the deviation consisted in a slight but definite increase in the content of the heavy isotope. The K contained in other tissues, i.e. liver, kidney, salivary gland and skeletal muscle, has occasionally shown minute deviations in an opposite direction.

In the living animal a continuous K exchange takes place between the various tissue cells and the blood plasma, the latter being probably in direct communication with the intercellular spaces. The exchange occurs in both directions, and generally at a slow rate. Further, the actual K content of the majority of tissues is very much (on an average perhaps twenty times) higher than that of the blood plasma, and this difference is maintained independently of the K exchange between cell and plasma. Although no definite picture of the mechanism of this exchange is available, it is certain that while K is specifically absorbed by the cell body, it remains in a state of dynamic equilibrium with the K in the surrounding medium. Under these conditions the appearance of an isotope effect will be possible.

A simple kinetic mechanism can be devised to account for the difference in the isotopic ratios of K in tissue (excluding bone marrow) and plasma. This mechanism depends on the difference in velocity of movement of the K atoms in the two media concerned. As far as we are aware, K atoms in the plasma and the medium directly surrounding the cell exist as free ions. The ratio of the numbers of $^{39}_{19}$K and $^{41}_{19}$K atoms striking the outer surface of the cell membrane, and entering thereby the cell, is then given by

$$n_{39}/n_{41} = \frac{C_{39}}{\sqrt{39}} \Big/ \frac{C_{41}}{\sqrt{41}}, \qquad \ldots\ldots(1)$$

where C_{39} and C_{41} denote molar concentrations of $^{39}_{19}$K and $^{41}_{19}$K in the plasma. Since a state of equilibrium exists, K atoms are also moving in the reverse direction. In the cell, in contrast to the plasma, K is associated with heavy molecular groups of mass M (possibly proteins); in consequence the ratio of the numbers of $^{39}_{19}$K and $^{41}_{19}$K atoms striking the inner surface of the cell membrane, and thereby leaving the cell, is expressed by

$$n'_{39}/n'_{41} = \frac{C'_{39}}{\sqrt{(M+39)}} \Big/ \frac{C'_{41}}{\sqrt{(M+41)}}, \qquad \ldots\ldots(2)$$

where C'_{39} and C'_{41} denote molar concentrations of $^{39}_{19}$K and $^{41}_{19}$K in the tissue. Since M is a mass large compared with that of the K atom, equation (2) may be written

$$n'_{39}/n'_{41} = C'_{39}/C'_{41}. \qquad \ldots\ldots(2a)$$

At equilibrium not only the total number of K atoms but also the ratio of the numbers of $^{39}_{19}$K and $^{41}_{19}$K atoms striking the cell membrane in the two directions must be the same. In consequence

$$\frac{C_{39}}{\sqrt{39}} \Big/ \frac{C_{41}}{\sqrt{41}} = C'_{39}/C'_{41}. \qquad \ldots\ldots(3)$$

From equation (3) the isotopic ratio of plasma K can be calculated if that of tissue K is given, and vice versa. If the value 14·20 is considered as the isotopic ratio of tissue K, the isotopic ratio of plasma K is found to be 13·85. This represents a 2·4 % increase in the $^{41}_{19}$K content of plasma K compared with that of tissue K (and mineral K), a figure which agrees well with the observed average increase of 2·5 %. The value 14·30 for tissue K would correspond to the value 13·95 for plasma K, representing a 2·3 % enrichment in $^{41}_{19}$K.

The mechanism described is proposed simply as a working hypothesis. Its claim to consideration rests on the fact that it accounts quantitatively for the observed isotope effect; while its validity depends on the condition that the intracellular K is completely associated with heavy molecules, and on the further condition that the hydration of the extracellular K ions can be neglected. If these conditions do not hold, the difference in the isotopic ratios calculable from equation (3) will be more or less reduced. Since this possibility exists, it is of interest to note that in addition to the kinetic mechanism a chemical mechanism may be in operation.

An appreciable fractionation of isotopes, due to slight differences in their chemical properties, can be obtained by means of 'exchange reactions' involving one or two phases, and a number of methods for isotope separation have been developed on this basis. A two-phase exchange reaction takes place, for instance, if the solution of an NH_4 salt is brought into contact with NH_3 gas. At equilibrium the ratios of the two N isotopes ($^{14}_{7}$N/$^{15}_{7}$N) in the liquid and gaseous phases were found to be as ~1 : 1·023, thus indicating a slight enrichment of the heavy isotope in the liquid compared with the gaseous phase [Urey, 1940]. Another kind of two-phase exchange reaction takes place if certain aluminosilicates known as zeolites, which normally contain Na as a basic constituent, are brought into contact with solutions of Li or K salts. The Na of the silicate is exchanged for Li or K, but the light isotope of Li and the heavy isotope of K are taken up preferentially. The deviations in the isotopic ratios obtained in this way were similar to the above [Taylor & Urey, 1938]. Correspondingly, the K normally present in another kind of zeolite can be exchanged for Na, the heavy K isotope being more difficult to replace than the light one [Brewer, 1939]. A certain fractionation of Li isotopes was further obtained by means of an exchange reaction between Li in alcoholic solution and Li present in Li-amalgam [Lewis & Macdonald, 1936]. Exchange reactions of these types, involving a liquid and a solid phase or two liquid phases (possibly water and lipoid), may occur in the process of K assimilation by the cell. Although the magnitude and direction of the isotope effect cannot be computed, differences approaching those observed may appear.

It remains to say that the actual values of the isotopic ratios of tissue K and plasma K do not depend solely on the mechanism responsible for their difference, but also on the isotopic ratio of the K which enters the blood stream by way of the intestine, and on the ratio of the total amount of K in tissue and plasma. Assuming that K when passing into the blood has an isotopic ratio close to 14·20, and considering the fact that the total amount of tissue K is several hundred times greater than the total amount of plasma K, the observed constancy or very slight increase in the isotopic ratio of the former and the marked decrease in the isotopic ratio of the latter are to be expected.

SUMMARY

The isotopic constitution of K present in various rat tissues, including red blood corpuscles and blood plasma, was studied as far as the two main isotopes $^{39}_{19}\mathrm{K}$ and $^{41}_{19}\mathrm{K}$ are concerned. It was found that the isotopic ratio $^{39}_{19}\mathrm{K}/^{41}_{19}\mathrm{K}$ in the ashes from bone marrow and blood plasma was distinctly lower than that of ordinary mineral K as contained in KCl (A.R.), and the content of the heavy isotope $^{41}_{19}\mathrm{K}$ was accordingly increased. K present in ash samples from other tissues showed, on the whole, the same isotopic ratio as mineral K, although a minute increase was found in exceptional cases. A kinetic mechanism has been proposed, which accounts for the difference in the isotopic ratios of tissue K and plasma K.

REFERENCES

Brewer (1936). *J. Amer. chem. Soc.* **58**, 365.
—— (1937). *J. Amer. chem. Soc.* **59**, 869.
—— (1938). *J. industr. engng Chem.* **30**, 893.
—— (1939). *J. Amer. chem. Soc.* **61**, 1597.
Lasnitzki & Brewer (1938). *Nature, Lond.*, **142**, 538.
—— & Oeser (1937). *J. chem. Soc.* p. 1090.
Lewis & Macdonald (1936). *J. Amer. chem. Soc.* **58**, 2519.
Taylor & Urey (1938). *J. chem. Phys.* **6**, 429.
Urey (1940). *J. Wash. Acad. Sci.* **30**, 277.

16. THE BIOCHEMISTRY OF SILICIC ACID

9. ISOLATION AND IDENTIFICATION OF MINERALS IN LUNG RESIDUES AND AIR-BORNE DUSTS FROM COAL MINES

BY G. NAGELSCHMIDT AND E. J. KING

From the Rothamsted Experimental Station, Harpenden, and the British Postgraduate Medical School, London

(*Received 30 December 1940*)

THE aetiology of the occupational lung affections described as silicosis, anthracosilicosis or more generally as pneumoconiosis is incompletely understood and a close comparison between the composition of the inhaled dusts and the minerals found in the affected lungs may be a useful preliminary to further knowledge. As most of the mineral particles in the lungs are below 5μ in diameter, identification with the petrological microscope is uncertain and X-ray diffraction apparatus becomes a necessary aid to the microscope. The qualitative knowledge of the minerals present, obtained from X-ray diffraction data, can be implemented by calculation of the minerals based on chemical or spectroscopic analysis.

It is intended in the present communication briefly to describe the methods used for the isolation of minerals from the lung tissue and for the X-ray diffraction analysis of these residues. The removal of coal and of acid-soluble material from air-borne dust from coal mines is also described, and data for a lung residue and an air-borne dust are presented. The chief minerals so far identified in this work are quartz, a member of the mica group and a member of the kaolin group.

Preparation of lung residues

Several methods have been proposed for preparing lung residues for X-ray diffraction work, and in some cases the same residues have been used for microscopic, chemical or spectroscopic analysis.

(1) *Simple drying of formalin-hardened lung tissue.* This method has been used by Sweany *et al.* [1938]: it has the advantage that the minerals are less altered than in any other method, but they are very much diluted by organic tissue and the resulting diagrams are poor (cf. Pl. 1 A).

(2) *Destruction of organic material by digestion with acid.* HNO_3 or other acid mixtures have been employed, usually followed by ignition at 600–700° [Jones, 1933; Kahane & Antoine, 1936; Jephcott *et al.* 1938; Burke & Kerr, 1938]. It gives a residue well suited for X-ray diffraction analysis, but it is always possible that minerals have been destroyed or altered by the heat treatment or by the acid attack. Burke & Kerr give evidence that even such a stable mineral as sericite shows after the treatment an X-ray diagram in which the lines are displaced against the lines of the untreated mineral. Any kaolinite present in the lung residues examined by the above investigators would have been destroyed by the heat treatment alone, as this mineral is destroyed by heating above 510°.

(3) *Destruction of organic material by enzymes.* Hicks *et al.* [1937] used this method. It is, however, slow, only part of the tissue can be removed and it is not easily applied to formalin-hardened tissue.

(4) *Removal of organic material by H_2O_2.* This method, described by Sundius *et al.* [1936] and recently used by Gärtner [1939], is in our opinion the best available, as it avoids the action of high temperatures and strong acids. As modified for the purposes of the present investigation, there are three stages in the treatment:

1. Removal of fatty material from powdered oven-dry lung with acetone.
2. Digestion with H_2O_2.
3. Extraction of the residue by acid or buffer solution.

The object of the third stage is to remove the endogenous inorganic constituents of the lung, mainly Ca, Fe, K, Na and P, as completely as possible with minimum alteration of the minerals, which form the dust in the lung. It may in some cases be impossible to attain both objectives quantitatively, but a fairly good separation can be made with repeated extraction with hot disodium hydrogen citrate at *p*H 6, or by brief treatment with cold *N* HCl. We have found that even prolonged treatment with *N* HCl or 2*N* HCl does not affect the appearance of X-ray diffraction diagrams of lung residues or mine dusts, as far as the diffraction lines of quartz or silicates of the kaolin and mica groups are concerned. That some small destruction of the quartz or more likely of the layer lattice silicates does take place is shown by the fact that SiO_2 is found in solution in the HCl extract, but this represents only a few per cent of the total silica in the dust or lung residue and would therefore not be reflected in X-ray diagrams with our present technique. Some results illustrating the extent of decomposition of mineral dusts by cold *N* HCl are shown in Table 1. With lung residues, decomposition and losses with cold *N* HCl and with hot disodium hydrogen citrate solution (*p*H 6) were slight. The amounts of SiO_2 in solution in the combined extraction and wash fluids have never exceeded a few mg., and much of this is derived from the amorphous silica of the lung tissue. The amounts of dissolved SiO_2 found in the cold HCl extracts have not been greater than those in the hot citrate extracts.

Table 1. *Treatment of powdered minerals and of air-borne dusts from coal mines with HCl*

1 g. powder shaken with 50 ml. *N* HCl at room temperature for 5 hr.

	mg. SiO_2 dissolved	Decomposition %*
Powdered minerals:		
Quartz	0·33	0·03
Kaolin	0·34	0·07
Mica sericite (Ohio)	1·81	0·45
Mica illite (S. Wales)	4·65	1·0
Air-borne dusts:		
2H2 (7·2% SiO_2)	1·26	1·8
2M1 (6·9% SiO_2)	1·22	1·8
1S1 (2·9% SiO_2)	0·48	1·7
1U1 (6·4% SiO_2)	0·55	0·9

* 100 × Dissolved SiO_2/Total SiO_2 per 1 g. powder.

The lung residues obtained by the H_2O_2 method may, however, contain large amounts of carbon (up to 80%), and the same applies to dusts collected in coal mines. Such carbon can be removed by prolonged heating of the samples to

380° in an electric muffle. This temperature is sufficiently high to oxidize the carbon in 40–80 hr. and at the same time low enough to avoid destruction of the kaolinite lattice. Pyrites and other sulphides and hydroxides which may be present in air-borne dusts would be oxidized or dehydrated by this treatment, and they should either be centrifuged out of the dust by means of heavy liquids before heating, or identified by the X-ray diagrams of the corresponding oxides, which would be present in the heated but not in the unheated material.

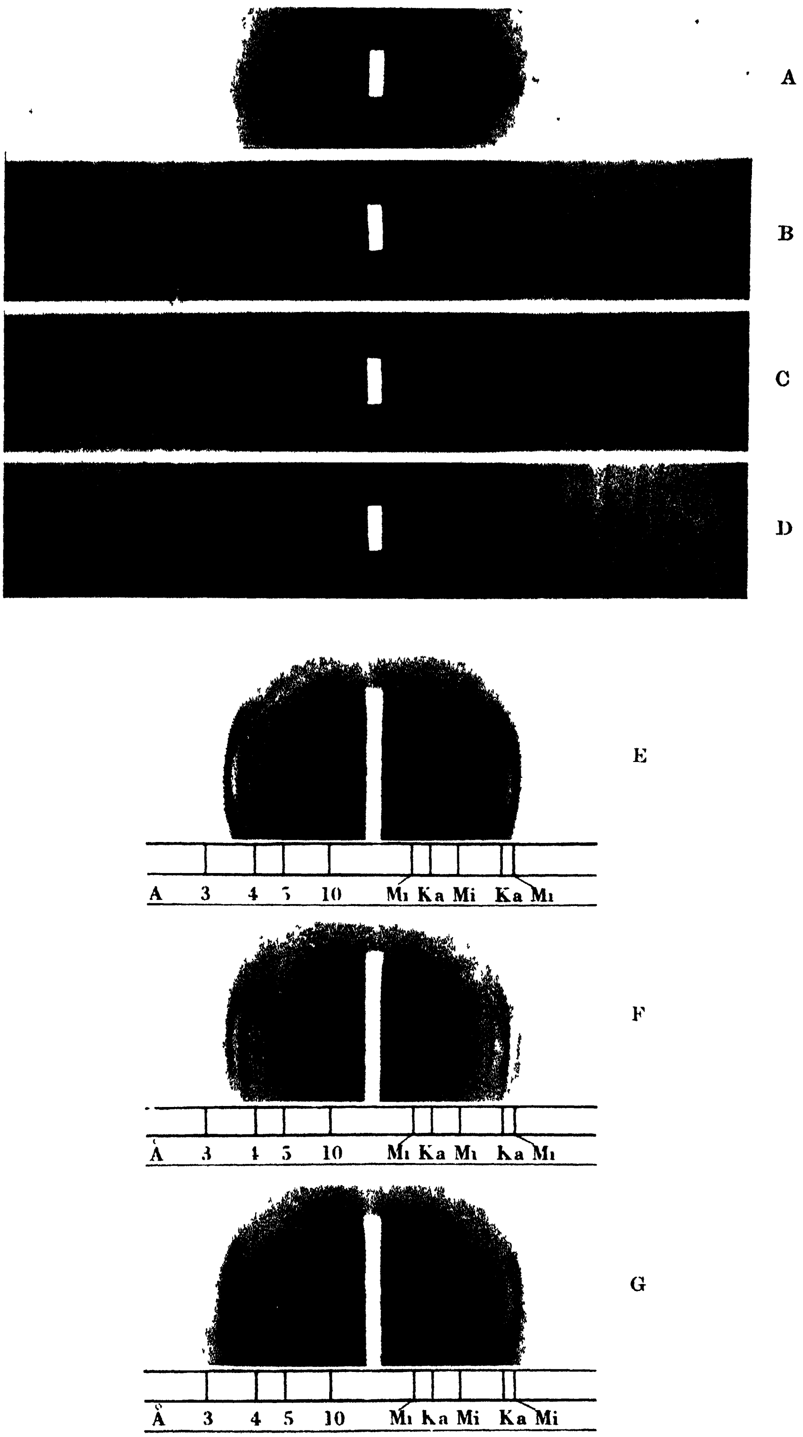
X-ray diffraction methods

In addition to taking X-ray powder diagrams in a cylindrical camera, we have used the aggregate method which has recently been described [Nagelschmidt, 1939] in its application to soil colloids. This method is specially suitable for the identification of layer lattice silicates such as mica, kaolinite, chlorite and montmorillonite in mixtures with each other. Ordered aggregates are made by slow evaporation of a suspension of the sample to be examined, and the aggregates are mounted similarly to single crystals in an X-ray spectrometer. The particles in the aggregate are oriented with their cleavage planes parallel to the plane of sedimentation and a set of good X-ray reflexions from the basal planes is therefore obtained. These basal reflexions are the main X-ray diffraction characteristics for the different layer lattice silicates. Kaolinite has strong basal reflexions at 7·14 and 3·57 Å., and mica has strong reflexions at 10 and 3·34 Å. and a weaker one at 5 Å. When aggregates of mixtures of these two minerals are heated to 510°, only the mica reflexions remain; the kaolinite reflexions are absent, as kaolinite is destroyed by heating to 500°. Chlorite shows strong reflexions at 14·1, 7·05, 4·70 and 3·52 Å. and these reflexions are still present in aggregates heated to 510°. The aggregate method is used in addition to, but not as a substitute for, the powder method. Powder and aggregate diagrams of a lung residue and a mine dust are shown in Pl. 1.

X-ray diffraction diagrams of lung residues and mine dusts indicate qualitatively the presence of different minerals if these minerals occur in sufficient amounts. Whether and, if so, how far both the powder and aggregate methods can be developed quantitatively is at present unknown. The quantitative estimation of quartz in mine dusts has been described by Clark & Reynolds [1936], but as it is based on the intensity of the 3·34 Å. reflexion of quartz it cannot be used in the presence of mica, which gives a strong reflexion for the same spacing. The method can, however, be modified, so as to be applicable to mixtures containing quartz and mica.

Preparation details

The lung is dried at 105° and reduced to a fine powder. 20 g. of the dried substance are extracted in a Soxhlet overnight with acetone to remove fatty material. The extraction thimble with its contents is dried in an oven; the contents of the thimble are knocked into a 2 l. pyrex beaker; 30 % H_2O_2 is added to a depth of about 1 in., and the mixture is carefully warmed on a water bath and stirred with a glass rod. If the froth tends to rise to the rim of the beaker, it can be whipped down with the stirring rod and if necessary octyl alcohol is added. As the frothing subsides, and as the liquid evaporates, more H_2O_2 is added. The depth of the liquid in the beaker should not exceed about 1 in., otherwise the mixture will tend to froth over. The additions of H_2O_2, with subsequent evaporations almost to dryness, should be continued until the additions no longer cause frothing. At this point the destruction of the lung tissue seems to be complete and any black material remaining is probably coal dust.


A
B
C
D
E
Å 3 4 5 10 Mi Ka Mi Ka Mi
F
Å 3 4 5 10 Mi Ka Mi Ka Mi
G
Å 3 4 5 10 Mi Ka Mi Ka Mi

X-Ray diffraction diagrams of a lung residue and a mine dust. A–D, Powder diagrams; E–G, aggregate diagrams. A, Oven-dry lung tissue; B, lung residue, 380° ash; C, mine dust, 380° ash after acid washing; D, mine dust, 380° ash before acid washing; E, lung residue, 380° ash; F, mine dust, 380° ash; G, same as F after heating to 520°. For reproduction, intensities in the centres of A–D have been reduced by partial shading.

The whole process, in our experience, takes about a week. The contents are brought nearly to dryness each evening. Each morning, and as often during the day as the mixture comes to dryness, more H_2O_2 is added.

The final residue consists of the inorganic salts from the lung tissue plus the dust. The former are brought into solution by extracting the mixture with acid. We have used *N* HCl with 15 min. vigorous shaking at room temperature, and hot *M*/10 citrate buffer of *p*H 6 with continuous stirring for 1 hr. On allowing the mixture to settle, the supernatant liquid is seen to contain much of the iron in solution. Separation of the solid from the liquid can be accomplished by gravity filtration through a fine paper (a hardened filter paper seems to retain the dust particles), or by prolonged centrifuging. We have found it convenient to transfer the residue, with washing, to 250 ml. centrifuge bottles, and to use 200 ml. portions of liquid for each extraction. Each separation is carried out by centrifuging for 1 hr. at 3500 r.p.m.

The residues are re-extracted several times to remove all salts. Iron is the most difficult of the tissue constituents to eliminate from the residue, and it is usually necessary to extract the centrifuged deposits three or four times with cold HCl or hot citrate buffer. The presence of iron in the extraction fluids may be tested for by treating a sample with acetic acid, sodium acetate and ferrocyanide.

The filtrate or supernatant fluid should be checked for the absence of dust particles by microscopic examination, and by colorimetric analysis for total SiO_2 [King, 1939]. The presence of appreciable numbers of dust particles will indicate the necessity for more prolonged centrifuging. With the residues we have handled, 1 hr. spinning appears to have been sufficient. The presence of non-particulate SiO_2, i.e. dissolved SiO_2, in any quantity indicates a decomposition of the siliceous matter by the extraction fluids. The residue is finally washed once with water and once with alcohol and is dried in an air oven.

Results

As an example, we give full details for one lung residue and one mine dust. The lung was kindly supplied by Dr P. D'Arcy Hart and the mine dust was collected by Dr P. F. Holt, working with Prof. H. V. A. Briscoe. The occupational history of the case from which the lung residue was taken will be given elsewhere, in conjunction with the results obtained in the study of a large group of workers.

Lung residue. The lung residue prepared in this way lost 12% of the dry weight on prolonged heating to 380°. Powder and aggregate X-ray diffraction diagrams of the 380° ash taken with unfiltered iron K radiation are shown in Pl. 1, B and E. A powder diagram of the untreated oven-dried powdered lung tissue is shown in Pl. 1, A. Table 2 gives the interplanar spacings and intensities of diffraction lines of the 380° ash diagram, and the interpretation in terms of quartz, mica and kaolin. There are many diffraction lines of quartz which do not coincide with other lines in the diagram, but only two lines, the basal reflexions, which can be ascribed to kaolin alone. It is not possible to distinguish under these conditions which member of the kaolin group it is; it can only be said that a member of the group is present. The kaolin group contains the minerals kaolinite, nacrite, dickite, anauxite and halloysite. No other mineral is known to give strong basal reflexions at 7·1 and 3·55 Å. spacings, which disappear if the sample is heated to 510°. For the mica it is likewise not possible to determine which member of the mica group is present. The diagram does not seem to correspond to well-crystallized muscovite or hydromuscovite, but rather to the type of mica found in shales and soil colloids. The term 'illite' [Grim *et al.* 1937] has been proposed for mica of this type, but samples described as sericite or secondary muscovite may refer to similar material.

Table 2. *X-ray data for lung residue, 380° ash*

d = interplanar spacings, Int. = intensities of diffraction lines, v.s. = very strong, s. = strong, m. = medium, w. = weak, v.w. = very weak. Q. = quartz, Mi. = a member of the mica group, Ka. = a member of the kaolin group.

} denotes edges of a band or two lines of different intensity which are not clearly separated.

No.	*d*	Int.	Mineral	No.	*d*	Int.	Mineral
1	10·1	s.	Mi.	13	1·815	s.	Q.
2	7·1	s.	Ka.	14	1·675} 1·644}	m.	Mi., Ka., Q.
3	5·0	w.	Mi.	15	1·539	m.	Q.
4	4·48}	s.	Mi., Ka.	16	1·508} 1·488}	s.	Mi., Ka.
5	4·23}	m.	Q.	17	1·382} 1·372}	s.	Q.
6	3·56	m.	Ka.	18	1·294} 1·280}	w.	Mi., Ka.
7	3·35} 3·31}	v.s.	Q., Mi.	19	1·200	m.	Q.
8	2·58} 2·54}	s.	Mi., Ka.	20	1·180	m.	Q.
9	2·46	v.w.	Mi., Q.	21	1·151	w.	Q.
10	2·45} 2·18}	v.w.	Ka., Q., Mi.	22	1·076	s.	Q.
11	2·181	m.	Q., Mi.				
12	2·003} 1·972}	m.	Mi., Ka., Q.				

Table 3 gives the chemical analysis of the lung residue carried out on the 380° ash. The water lost below 105° shown in Table 3 has been reabsorbed from the air by the ash. It will be seen that SiO_2, Al_2O_3, K_2O, and water above 105°, account for 94 % of the residue, and these four are the main chemical constituents of a mixture of quartz, muscovite and kaolin. It is possible to calculate the relative amounts of the three minerals in the lung residue if it is assumed that all the SiO_2, Al_2O_3 and K_2O are present in these minerals, and if the composition of the muscovite and of the kaolin are known. Such calculations with kaolin and muscovite of theoretical composition give for the lung residue: muscovite 26 %, kaolin 46 % and quartz 24 %. The chemical composition of mica may vary within wide limits, and the mica found in shales and soil colloids usually contains only 4–6 % K_2O, as against muscovite with 11·8 % K_2O. Calculation of the minerals in the lung residue with a mica containing 5 % K_2O, 48 % SiO_2 and 32 % Al_2O_3, and kaolin of theoretical composition, gives for the

Table 3. *Chemical analysis of lung residue ashed at 380°*

Analyst: Chemical Laboratories, London

SiO_2	56·9
Al_2O_3	28·3
TiO_2	1·4
Fe_2O_3	1·5
MgO	0·7
CaO	0·5
Na_2O	0·8
K_2O	3·1
H_2O, above 105°	5·4
H_2O, below 105°	1·1
P_2O_5	0·1
	99·8

residue a mixture of 62 % mica, 21 % kaolin and 17 % quartz. The X-ray diffraction data indicate only that all three minerals occur as main constituents. Their true relative proportions probably lie between the two sets of percentages given above. If special interest is centred on SiO_2, it will be seen that less than half of the total SiO_2 shown in the analysis, or less than a quarter of the mineral residue, occurs as quartz.

Microscopic inspection of the 380° ash of the lung residue shows that nearly all particles are well below 2 μ in diameter and it does not seem possible to distinguish the different constituents quantitatively by their refractive index or other optical characteristics with microscopic petrological methods.

Mine dust. The untreated mine dust gave a poor X-ray diffraction diagram. After heating the material to constant weight at 380° a residue consisting of 15·1 % of the original dust was obtained. X-ray powder and aggregate diagrams of this residue are shown in Pl. 1, D and F, and a diagram of the same residue after a short treatment with *N* HCl in Pl. 1, C. Pl. 1, G shows an aggregate diagram, taken with the same specimen as Pl. 1, F, after heating it to 540° for 24 hr. The kaolin lines are absent. Table 4 gives the evaluation of the diagram shown in Pl. 1, C. It is again only possible to state that quartz and members of the kaolin and mica groups are present. The presence of a line at 14·3 Å. on aggregate diagrams may possibly indicate minor amounts of chlorite. The diagram before the *N* HCl treatment shows a few additional diffraction lines indicating dolomite or ankerite. Dolomite is a calcium-magnesium carbonate and ankerite a calcium-magnesium-iron carbonate.

Table 4. *X-Ray data for mine dust, 380° ash, after treatment with* N *HCl*

d = interplanar spacings, Int. = intensities of diffraction lines, v.s. = very strong, s. = strong, m. = medium, w. = weak, v.w. = very weak. Q. = quartz, Mi. = a member of the mica group, Ka. = a member of the kaolin group.

} denotes edges of a band or two lines of different intensity which are not clearly separated.

No.	*d*	Int.	Mineral	No.	*d*	Int.	Mineral
1	10·2	m.	Mi.	13	1·540	m.	Q.
2	7·1	v.s.	Ka.	14	1·508 1·493 1·484	m.	Mi.
3	4·48	v.s.	Ka., Mi.	15		s.	Ka.
4	3·55	s.	Ka.	16	1·380 1·370	s.	Q.
5	3·35 3·31	v.s.	Q., Mi.	17	1·318	w.	?
6	2·58 2·55	s.	Ka., Mi.	18	1·286	w.	Ka., Mi.
7		v.w.	Mi., Q.	19	1·255	w.	Q.
8	2·37 2·33	m.	Ka.	20	1·236 1·225	v.w.	Ka., Mi.
9	2·131	w.	Q., Mi.	21	1·200	m.	Q.
10	1·998 1·980	m.	Ka., Mi., Q.	22	1·180	m.	Q.
11	1·816	m.	Q.	23	1·151	w.	Q.
12	1·671 1·642	s.	Ka., Mi., Q.		1·076	m.	Q.

Table 5 gives the chemical analysis of the 380° ash of the mine dust. SiO_2, Al_2O_3, K_2O and water above 105° account for 86 % of the ash and most of the remaining Fe, Ca and Mg is present as carbonate, sulphate or oxide, although a proportion of these may form chlorite with some of the SiO_2 and Al_2O_3. The mineral content has been calculated ignoring chlorite and assuming all SiO_2, Al_2O_3 and K_2O to be present as quartz, kaolin or muscovite of theoretical composition. The mine dust ash contains according to this calculation 14 % muscovite, 66 % kaolin and 11 % quartz. Assuming a mica containing 5 % K_2O,

Table 5. *Chemical analysis of mine dust ashed at 380°*

Analyst: Geochemical Laboratories, London

SiO_2	47·9
Al_2O_3	31·6
TiO_2	0·8
Fe_2O_3	3·9
MgO	1·4
CaO	3·1
Na_2O	1·0
K_2O	1·6
H_2O, above 105°	4·6
H_2O, below 105°	0·7
CO_2	0·8
SO_3	2·2
S	0·1
	99·7

48 % SiO_2 and 32 % Al_2O_3 the mine dust would contain: 32 % mica, 54 % kaolin, 8 % quartz. The water above 105° shown in Table 5 is too low to satisfy the amount of lattice hydroxyl present in kaolin of theoretical composition for either of these mixtures, but many kaolin samples contain less than the theoretical amount of water and part of the lattice hydroxyl may have been removed by the initial prolonged heating to 380°.

Free SiO_2 (quartz) [Shaw, 1934] amounted to 12 % of the mine dust ash, in fair agreement with the estimate of 11 % from the calculation with muscovite and kaolin of theoretical composition. We are grateful to Mr A. Shaw for carrying out this determination.

SUMMARY

The isolation of mineral residues from lungs by the H_2O_2 method and the removal of coal from such residues and from air-borne dusts from coal mines by heating to 380° are described. X-ray diffraction methods are used for the identification of minerals in the residues and full data, including chemical analyses, presented for a lung residue and a mine dust. Quartz, a member of the kaolin group and a member of the mica group are the main constituents found in the lung residue and in the air-borne dust.

Grateful acknowledgement is made to Dr A. Brammall for his advice and active interest in this investigation. We are indebted to the Medical Research Council for a grant covering part of the expenses of this investigation.

REFERENCES

Burke & Kerr (1938). *J. industr. Hyg.* **20**, 535.
Clark & Reynolds (1936). *J. industr. Engng Chem.* Anal. Ed. **8**, 36.
Gärtner (1939). *Arch. Gewerbepath. Gewerbehyg.* **9**, 377, 634.
Grim, Bray & Bradley (1937). *Amer. Min.* **22**, 813.
Hicks, McElroy & Warga (1937). *J. industr. Hyg.* **19**, 177.
Jephcott, Gray & Irwin (1938). *Canad. med. Ass. J.* **38**, 209.
Jones (1933). *J. Hyg., Camb.*, **33**, 307.
Kahane & Antoine (1936). *Bull. Soc. Chim. biol., Paris*, **18** ,1769.
King (1939). *Biochem. J.* **33**, 944.
Nagelschmidt (1939). *J. agric. Sci.* **29**, 477.
Shaw (1934). *Analyst*, **59**, 446.
Sundius, Bygden & Bruce (1936). *Trans. ceram. Soc.* **35**, 167
Sweany, Klaas & Clark (1938). *Radiology*, **31**, 299.

17. STUDIES ON DIFFUSING FACTORS

THE HYALURONIDASE ACTIVITY OF TESTICULAR EXTRACTS, BACTERIAL CULTURE FILTRATES AND OTHER AGENTS THAT INCREASE TISSUE PERMEABILITY

By D. McCLEAN
With the assistance of C. W. HALE
From the Lister Institute of Preventive Medicine, Elstree, Herts

(*Received 30 December 1940*)

AQUEOUS extracts of mammalian testicle contain a factor that dramatically increases the permeability of the tissues to injected fluids [McClean, 1930; 1931; Hoffman & Duran-Reynals, 1931]. This can be demonstrated by the rapid disappearance of the bleb following intracutaneous injection of these extracts and by the rapid spread through a large area of skin of any suitable coloured indicator that may be injected together with them. This factor is associated with the germinal epithelium and can be extracted from spermatozoa [McClean, 1931]. Factors with similar diffusing properties have been obtained from the most diverse sources, for example, from filtrates and extracts of staphylococcus and streptococcus [Duran-Reynals, 1933], from organisms of the gas-gangrene group and virulent pneumococci [McClean, 1936], from extracts of malignant tissues [Duran-Reynals & Stewart, 1931; Boyland & McClean, 1935], from snake and spider venoms [Duran-Reynals, 1939] and from leeches [Claude, 1937].

Various methods for the partial purification of testicular extracts and of bacterial culture filtrates of the gas-gangrene group have been described [Morgan & McClean, 1932; Favilli, 1936; Claude & Duran-Reynals, 1937; McClean, 1936; Madinaveitia, 1938; 1939, 1, 2; Mannozzi-Torini, 1939], but no naturally occurring diffusing factor has been isolated in the pure state. Nobody had succeeded in elucidating the mechanism whereby this diffusion effect is produced until Chain & Duthie [1939] reported that purified preparations of testicular diffusing factor exhibit a remarkable mucolytic activity characterized by a rapid fall in the viscosity of mucoprotein preparations and the liberation therefrom of reducing substances. This observation suggested that the mucolytic and diffusing activities might be due to the same factor and that the spread in the tissues might be due to the action of the enzyme on the mucin-like interfibrillar substance in the collagen of the dermis.

It was obviously important to confirm the observations of Chain & Duthie, to determine whether diffusing factors from other sources exhibit a similar mucolytic activity and to make a comparative study of these enzymes. Madinaveitia & Quibell [1940] have already described a viscosimetric method whereby testicular mucinase may be accurately estimated and they have investigated many of the characters of the reaction. During the progress of our own observations Meyer *et al.* [1940, 1] reported the hydrolysis of hyaluronic acid by bacterial enzymes; it had previously been shown by Meyer & Palmer [1936] that the mucoproteins of vitreous humour and umbilical cord contain a mucopolysaccharide which they had designated by the term hyaluronic acid. More recently

Robertson *et al.* [1940] described an enzyme derived from culture filtrates of *Cl. welchii* and other organisms which hydrolyses synovial fluid; neither these authors nor Meyer and his co-workers had at that time connected the mucolytic activity of their preparations with the action on the permeability of the tissues. Subsequently, however, Meyer & Chaffee [1940] published a preliminary account of the composition of a polysaccharide obtained from the cornea and a comparison of its properties with the polysaccharides obtained from vitreous humour and synovial fluid which they consider to be identical with it and which are hydrolysed by the same enzyme preparations. They suggested that the substrate in the skin upon which the enzyme acts to produce the spreading effect may not be hyaluronic acid itself but its sulphuric acid ester. Meyer *et al.* [1940, 2] have since published a note on the relationship between 'spreading factor' and 'hyaluronidase'. They found that all preparations containing hyaluronidase also exhibit spreading properties and that both factors possess attributes in common. They have succeeded in isolating a polysaccharide from the skin which is hydrolysed by hyaluronidase. On the other hand, these authors state that a number of preparations which possess marked spreading action do not hydrolyse hyaluronic acid or reduce the viscosity of solutions containing the polysaccharide. They also reported that antisera prepared against pneumococcal hyaluronidase, which completely neutralize the hydrolytic activity, fail to inhibit the spreading activity of the pneumococcal preparations. This result is not in agreement with ours obtained with antisera prepared against the purified diffusing and hyaluronidase factors from *Cl. welchii* and *Vibrion septique*; our results will be described in the course of this paper, but it may be suggested here that the failure of Meyer and his co-workers to neutralize the spreading activity might be due to failure to balance the dose of the factor against that of the antiserum; one of us [McClean, 1936] has shown that the most potent antisera then obtainable would only neutralize a limited number of minimal diffusing doses of the spreading factor.

Favilli [1940] reported that several snake venoms which possess high diffusing activity also show a marked mucolytic effect and that this effect is inhibited by appropriate antisera. This author also reported that the diffusing extracts from leeches exhibit mucolytic activity. Claude [1937] had reported the diffusing activity in the skin of leech head extracts and he [1940] confirmed the mucolytic activity of these extracts and noted a parallelism between the mucolytic and spreading powers of various extracts. He also prepared a mucoprotein from normal rabbit skin, the viscosity of which is rapidly reduced by leech extracts, and concluded that the spreading action in the skin was due, at least in part, to the power of these extracts to cause hydrolysis or depolymerization of the same compound *in vivo*. Claude [1935] had previously reported that azoproteins diffused in the skin in a manner similar to, though with less rapidity than testicular extracts; Favilli showed that these diazotized compounds cause a marked fall in the viscosity of mucoprotein preparations, but he was unable to decide whether reducing substances were liberated as a result of this action.

Our own observations have been designed to study the characteristics of the hyaluronidase activities of extracts from testis, from various bacteria and from other sources in order to determine how far the enzymes are identical in their properties. We have also attempted to correlate the hyaluronidase and spreading activities of these preparations in order to determine whether diffusion in the tissues may be due to the action of hyaluronidase. Our observations have led us to investigate the relationship between activities of hyaluronidase and of other enzymes such as phosphatase and amylase present in the bacterial filtrates. We have also made some preliminary observations on the part played by hyaluronidase in the metabolism of certain bacteria with a view to elucidating the activity of this enzyme in the tissues during infection. Some of the observations to be described in this paper have already been briefly reported in preliminary communications [McClean & Hale, 1940, 1, 2].

Methods

Preparation of substrate. In preliminary experiments vitreous humour was used as substrate, but, in view of its spontaneous loss of viscosity on storage, to which reference will be made later, this substance was replaced by preparations of mucoprotein from umbilical cords. The cords were stored in acetone and minced as required. 100 g. of the dried powder were extracted with 1 l. of distilled water for 24 hr. in the refrigerator and this extraction was repeated once. The fluid was squeezed through twill and both supernatants combined. The aqueous solution was purified by the method described by Meyer & Palmer [1936] which depends upon precipitation with ice-cold alcohol. We avoided the use of glacial acetic acid in this method in order to preserve the viscosity of the mucoprotein; flocculation of the precipitate was accelerated by the addition of a few ml. of saturated alcoholic sodium acetate solution. The final precipitate was washed with acetone and dried over P_2O_5 *in vacuo*. About 3 g. of a white fluffy powder can be obtained from 100 g. of dried umbilical cord. This powder contains about 7 % total N and a 0·25 % solution of it in water has a relative viscosity of about 400 compared with water as 100. Aqueous solutions were preserved by the addition of a few drops of chloroform and were kept in the refrigerator. A very slow loss of viscosity occurs. This preparation was used for most of our experiments.

For some observations, it was desired to use a purified salt of hyaluronic acid as substrate. For this purpose the preparation described above was further purified by the method of Robertson *et al.* [1940], which depends upon tryptic digestion of the protein, precipitation of this with trichloroacetic acid and final precipitation of the polysaccharide with alcohol as a potassium salt. About 350 mg. of this powder are obtained from 1 g. of the mucoprotein. 0·2 % aqueous solutions of the polysaccharide have a relative viscosity of about 350. The powder contains about 3 % total N and 0·5–1·0 % P.

Preparation and purification of enzymes. Samples of testicular enzyme purified by methods described by Morgan & McClean [1932] and by Madinaveitia [1938; 1939, 1, 2] for the purification of diffusing factor were used. Unpurified bacterial culture filtrates were employed as well as samples from *Cl. welchii* filtrates purified by the method described by McClean [1936], which depends upon precipitation of the active fraction with basic lead acetate. Filtrates from *Cl. oedematis maligni* (*Vibrion septique*) were purified with alumina $C\gamma$ by the method used by Madinaveitia [1939, 2] for *Cl. welchii* filtrates and testicular extracts. In our experience it is impossible to purify *Vibrion septique* filtrates by the lead precipitation method successfully employed with *Cl. welchii.* Our observations with Russell's viper venom were made with the dried venom in the form of 'Stypven' which was kindly supplied by Dr Parish of the Wellcome Physiological Research Laboratories.

Viscosimetric technique. Madinaveitia & Quibell [1940] have shown that, under certain defined experimental conditions, the time taken by any sample of enzyme to reduce the viscosity of the substrate to a level half-way between its original level and that of the solvent is inversely proportional to the concentration of the enzyme. They have also shown that the reaction time, that is the time taken to reach half viscosity level, is independent of the concentration of the substrate and of its original viscosity. Both these facts have been confirmed by us and, for the purpose of this work, we have provisionally defined a viscosity-reducing unit (V.R.U.) as that concentration of enzyme which will reduce the viscosity of a standard substrate preparation to a level half-way between its original figure and that of the solvent employed in 20 min. The selection of this period of time is determined by experimental conditions that will be described. Madinaveitia & Quibell employed a final concentration of 0·66 *M* NaCl in their system. It appeared that the *M*/6 citrate buffer supplied sufficient electrolyte and, since we did not wish to work in a salt concentration so widely different from that present under biological conditions, we omitted the addition of NaCl in our earlier experiments. Later, for reasons that will appear in the text, we have added NaCl to a final concentration of 0·1 *M*.

Four vol. of 0·25 % mucoprotein or 0·2 % potassium hyaluronate in distilled water are mixed with 1 vol. of *M* sodium citrate buffer at *p*H 4·6. The buffer is prepared by mixing 1 vol. of *M* citric acid with 2 vol. of *M* NaOH. The mixture of substrate and buffer is placed in the water bath at 34°. 0·5 ml. of the appropriate dilution of the enzyme is also placed in the water bath in a separate tube. A similar dilution of the enzyme previously heated for 30 min. in boiling water serves as control. Two Ostwald viscosimeters with the same or closely similar flow times are placed in the bath; these and the sample of enzyme and substrate are left to warm for at least 10 min. After this interval 2·5 ml. of the buffer substrate mixture are run into each of the tubes containing heated and unheated enzyme. A stopwatch which is started at this moment will record the reaction time (*R*). The substrate, buffer and enzyme mixtures are mixed with warm pipettes and 1·5 ml. of each are run into the viscosimeters so that one viscosimeter contains the test mixture and the other the control. The fluid is blown (not sucked) into the bulb of the viscosimeter in order to avoid the formation of bubbles. The first reading is taken on another stopwatch 1 min. after mixing and thereafter at frequent intervals until the flow time of the unheated enzyme system is less than half the sum of that of the heated enzyme system and that of the solvent employed. The flow time readings on either side of the half viscosity level are plotted against time and the point at which the line crosses the half viscosity level is taken as the reaction time of the sample of enzyme under test. A correction is made for the fall in viscosity that occurs during a reading of the flow time; half the flow time is added to the time at the beginning of the run of the fluid through the viscosimeter. In order to facilitate calculation of relative viscosities, half viscosity levels and enzyme strength, all the flow times are considered to be relative to a flow time of 100 for *M*/6 sodium citrate buffer. Thus

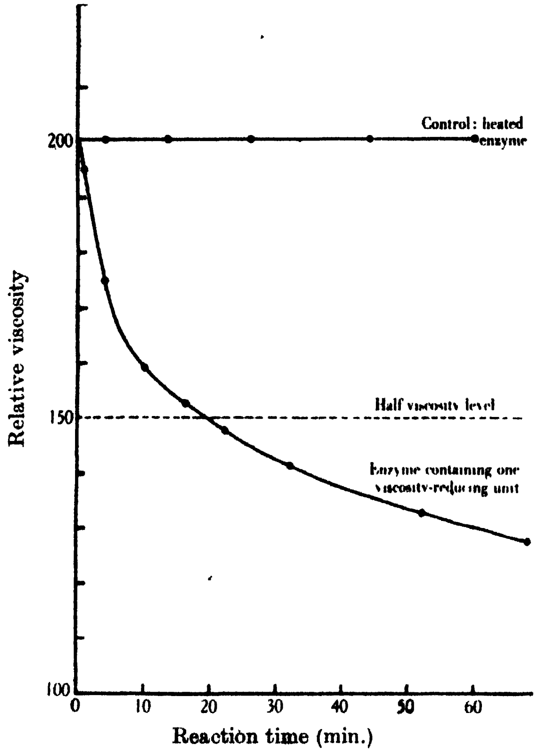


Fig. 1. Typical curve of enzyme containing one viscosity-reducing unit.

$$\frac{\text{Flow time of substrate}}{\text{Flow time of buffer}} \times \frac{100}{1} = \text{relative viscosity.}$$

Viscosimeters were closely matched and there were not more than 1 or 2 sec. difference in the time of flow of the buffer solution. The viscosimeters used gave a flow time of about 50 sec. for 1·5 ml. of this fluid. In order to compare the potencies of different enzyme samples accurately it is necessary to use dilutions which give reaction times falling between 15 and 45 min. If the experimental conditions described are carefully observed assays of the viscosity-reducing activity of enzymes can be made with an error of ±10 %. A typical calculation of the activity of a sample of enzyme is as follows:

Reaction time to half viscosity level $(R) = 30$ min.
Amount of dilution of enzyme sample $(D) = 60$ (dilution of 1 : 60).

$$\text{Then viscosity-reducing units (already defined)} = \frac{D \times 20}{R} = \frac{60 \times 20}{30} = 40.$$

A typical viscosity reduction curve for a sample containing 1 unit of activity is shown in Fig. 1.

Estimation of reducing substances. N-Acetylhexosamine was estimated by the method of Morgan & Elson [1934]. The colour produced in the test solutions was matched against standard solutions in an optical colorimeter. The method gave most accurate results when the concentrations of acetylhexosamine were between 20 and 200 μg./ml. Total reducing substances were estimated by the method of Hagedorn & Jensen.

Diffusion in the dermis. Three methods of assaying diffusing activity in the skin are available. The original method described by one of us [McClean, 1930; 1931] depends upon the determination of the highest dilution of a sample that will produce a significant (20 %) increase in the area of spread in the skin. Madinaveitia [1938] has described a method which depends upon the comparison of the rate of spread of the sample under test with that of a standard preparation of diffusing factor. He did not claim that it was possible to differentiate between less than tenfold dilutions of the diffusing factor by this method. Bacharach *et al.* [1940] have recently described a method for estimating testicular diffusing factor depending upon a comparison of the areas of spread after 20 min. of two dilutions of a sample solution with those of two dilutions of a standard preparation. The injections are so arranged on the shaved backs of four rabbits as to allow for corrections for variation in animal susceptibility to the diffusing factor. These authors claim to be able to distinguish between doses in the ratio of 2 to 1. An investigation is being made into the correspondence between determinations of diffusing activity made by the method of Bacharach *et al.*, and by the original method of determining the minimal diffusing dose; observations are also in hand to determine whether both methods are equally applicable to testicular, bacterial and other diffusing substances. Pending the result of this investigation it has seemed advisable to adhere to assay by the determination of the minimal diffusing dose for the purpose of the work to be reported in this paper. Notwithstanding the fact that we cannot claim to be able to distinguish between less than tenfold dilutions, we have noticed that the dose response curve does become steeper as the limiting dilution of the diffusing factor is being reached. There is therefore less liability to confusion between the areas of spread of two succeeding dilutions than when more concentrated solutions are employed. The unsatisfactory nature of this animal test from a quantitative point of view must be admitted, but it does suffice for the broad comparison of diffusing and hyaluronidase activities attempted in this work.

Tenfold dilutions of the sample to be tested were made in isotonic haemoglobin solution. 0·4 ml. samples of these dilutions were injected into the backs of clipped or depilated white-skinned rabbits. Two control injections of 0·4 ml. of haemoglobin solution alone were made into each rabbit. The length and breadth of the areas stained with haemoglobin were measured 20 min. after injection and the areas calculated on the assumption that they were ellipses according to the formula $\pi Dd/4$ when $D =$ the long axis and $d =$ the short one. The assumption that the area is elliptical is valid for the higher dilutions of the spreading factor only. Estimation of the area of spread of haemoglobin is facilitated if the animal's back is smeared with liquid paraffin before the measurements are made. Our results were expressed as percentage increase in area compared with that of the control injections of haemoglobin solution alone.

EXPERIMENTAL

Association between the hyaluronidase activities of various extracts and their diffusion in the dermis

The mucolytic activity of testis extract reported by Chain & Duthie was confirmed with mucoprotein from either vitreous humour or umbilical cord as substrate. Preparations of this diffusing factor purified either by the method of Morgan & McClean or by those of Madinaveitia showed similar activities in the reduction of the viscosity of the substrate and the liberation of reducing substances. Our preliminary experiments were designed to demonstrate a qualitative association between the diffusing and hyaluronidase activities of enzymes from various naturally occurring sources; no quantitative comparisons of the two activities were made at this stage.

Purified preparations of the diffusing factor from filtrates of *Cl. welchii* and *Cl. oedematis maligni* (*Vibrion septique*), which in high dilution cause rapid diffusion in the skin, also show marked hyaluronidase activity as demonstrated by viscosity reduction and the liberation of *N*-acetylhexosamine. These organisms are both members of the group of gas-gangrene-producing anaerobic organisms. Filtrates from certain strains of staphylococcus, streptococcus and from a virulent type 1 pneumococcus are known to diffuse actively in the skin; as will be seen in Table 1, these filtrates also showed marked hyaluronidase activity. On the other hand filtrates from a virulent strain of *S. typhi*, from *Cl. tetani* and from *C. diphtheriae* which produce no diffusion also contain no hyaluronidase.

Table 1. *The qualitative association between diffusion in the dermis and hyaluronidase activity*

	Diffusion in 20 min.			
Source of enzyme	Dose ml.	Increase in area %	Hyaluronidase units Undiluted sample	Liberation of *N*-acetyl-hexosamine
Testis extract (bulls)	4×10^{5}	84	90	+
Russell's viper-venom ('Stypven')	0·016	38	0·5	+
Cl. welchii filtrate (strain 107)	4×10^{-6}	35	70	+
Cl. oedematis maligni filtrate (*V. septique* KF 5)	4×10^{-5}	82	22	+
Staphylococcus filtrate (strain Wood 46)	4×10^{-2}	150	60	+
Pneumococcus filtrate (type 1 virulent)	4×10^{-2}	115	6	+
Streptococcus filtrate (5 strains)	4×10^{-2}	50–100	0·3	+
Streptococcus filtrate (3 strains)	4×10^{-2}	Nil	Nil	–
C. diphtheriae filtrate (strain 110)	0·2	Nil	Nil	–
Cl. tetani filtrate	0·2	Nil	Nil	–
S. typhi filtrate	0·2	Nil	Nil	–

Note. *Diffusion tests:* 0·4 ml. of a dilution of the sample to be tested was injected intracutaneously. The area of spread of these single injections is not an accurate expression of the relative diffusing activities of the different samples and no quantitative correlation between diffusing and hyaluronidase activities was attempted in this experiment.

The activity of the filtrate from virulent type 1 pneumococci is of interest, since one of us [McClean, 1936] has reported that the same strain rendered avirulent by cultivation in type-specific immune serum loses its diffusing power; on the other hand Meyer *et al.* obtained their hyaluronidase from the autolysate of a 'non-encapsulated non-type-specific variant' of a type 2 strain and drew a distinction between the enzyme obtained from pneumococci which is bound to the cell structure and that from *Cl. welchii* which is free in the fluid medium. It is clear from our observation that encapsulated virulent pneumococci excrete the enzyme into the medium in a manner similar to that of members of the gas-gangrene group.

It has already been mentioned in the introduction that several snake and spider venoms diffuse in the skin and that Favilli had reported that such snake venoms also showed a marked mucolytic activity. Through the courtesy of Dr Parish of the Wellcome Physiological Research Laboratories we were able to examine these properties in dried Russell's viper venom ('Stypven'). The results are summarized in Table 1. It will be seen that there is again an obvious association between diffusing and hyaluronidase activities. Further observations on the activity of this venom will be described later when the general properties of these enzymes are considered.

Certain artificially prepared compounds such as azoproteins and ascorbic acid and its oxidation products were found to diffuse in the skin and to reduce the viscosity of mucoprotein. Since, however, the characters of the diffusion and of the action on mucoprotein differ in many respects from those of the naturally occurring enzymes consideration of the behaviour of these substances will be postponed until that of the enzymes has been described.

Characters of the hyaluronidase activity of enzymes from various sources

Influence of pH. We have confirmed the result reported by Madinaveitia & Quibell that the action of the testicular enzyme on the viscosity of the substrate is greatest in the region of *p*H 4·6. The rate of the reaction decreases sharply on the acid side of this and more gradually as the *p*H approaches neutrality. Similar observations on enzymes obtained from culture filtrates of *Cl. welchii, Vibrion septique* and from Russell's viper venom indicate that they are all most active in the region of *p*H 4·6. All these enzymes show the same sharp decline in activity on the acid side of this *p*H, but whereas the enzyme from viper venom is completely inactive on the alkaline side of *p*H 6·5 those from *Cl. welchii* and *Vibrion septique* still cause a slow fall of viscosity at *p*H 8·5.

Liberation of N-*acetylhexosamine by these enzymes.* Preliminary observations on the rate of liberation of *N*-acetylhexosamine from mucoprotein by these enzymes indicated that, whereas in the presence of a sufficient concentration of enzyme the fall in viscosity may be so rapid that half viscosity level is reached in a few minutes, no acetylhexosamine can be detected for some hours. An experiment was performed in which purified potassium hyaluronate was exposed to the action of the purified *welchii* enzyme with the result shown in Table 2. It will be seen that although half viscosity level was reached in between 1 and 10 min. no measurable amount of acetylhexosamine could be detected for 50 min.; the maximum liberation was not attained until considerably after 2 hr. had elapsed. Similar results were obtained with the testicular enzyme.

This result indicated that two separate mechanisms might be involved and, furthermore, our early experiments suggested that enzymes from different sources might differ qualitatively as well as quantitatively in their capacities to liberate reducing substances. Thus it appeared that the bacterial enzyme

Table 2. *Time relation of viscosity reduction and liberation of* N-*acetylhexosamine*

Substrate: Potassium hyaluronate 2 mg./ml.
Enzyme: Purified from *Cl. welchii* filtrate.

Time Hr.	 Min.	Relative viscosity	*N*-acetylhexosamine
0	0	350	0
0	1	250	0
······	······	······	······
0	10	162	0
0	20	138	Trace
0	30	127	Trace
0	50	120	<0·1 mg./ml.
1	10	115	<0·1 ,,
2	0	108	0·1 ,,
18	0	102	1·0 ,,

Viscosity of buffer solution + enzyme = 100. The dotted line indicates the half viscosity level and the approximate time taken to reach it.

liberated more reducing substances than that from testis and that no reducing substance could be detected after the action of viper venom. When, however, the concentrations of the enzymes from different sources were adjusted so that their reaction times for the reduction of viscosity were comparable, it was found that the amount of enzyme necessary to liberate measurable quantities of reducing substances was approximately the same whatever the source of the enzyme. The result of this experiment is shown in Table 3.

Table 3. *Relation between* N-*acetylhexosamine liberation and viscosity-reducing activity of enzymes from various sources*

Substrate: Potassium hyaluronate 1·5 mg./ml.

Source of enzyme	Dilution containing 1 viscosity-reducing unit (V.R.U.)	Amount of *N*-acetylhexosamine (mg./ml.) liberated in 18 hr. by 60 V.R.U.	 6 V.R.U.	 3 V.R.U.
Cl. welchii (strain 107)	1 : 66	1·0	0·1	Trace
Staphylococcus (Wood 46)	1 : 60	0·8	0·1	Trace
Pneumococcus (type 1 virulent)	1 : 6·6	--	Trace	Trace
Russell's viper venom ('Stypven' 2 mg./ml.)	1 : 10		0·1	0·1
Testis (Madinaveitia standard prep. 10 mg./ml.)	1 : 5	—	0·4	0·2

Later observations indicated that there is not complete correlation between viscosity reduction and liberation of reducing substances by different samples of enzyme from the same source. Thus, in the experiment illustrated in Table 3, the testicular enzyme liberated at least four times as much acetylhexosamine as comparable concentrations of enzymes derived from bacteria and venom; in another experiment, with a sample of testicular enzyme purified by a different method, it was found to liberate less than half the amount of acetylhexosamine liberated by a *welchii* enzyme of the same viscosity-reducing activity. The time lag in the liberation of reducing substances, coupled with the lack of complete correlation between this activity and the reduction of viscosity, suggest that two separate mechanisms may be involved; if the enzymes are not identical, they must be very closely associated since they are not separated by any existing method of purification and, as will appear, they are similarly influenced by variations in the environment of the reaction.

Neutralization of enzyme activity by antisera. One of us [McClean, 1936] has already reported that antisera can be prepared which inhibit the spread of diffusing factors of bacterial origin in the dermis; it was also reported that the animals actively immunized against the purified diffusing factor in order to produce these antisera were themselves resistant to the diffusing activity of the homologous factor. Experiments were made to determine whether these sera

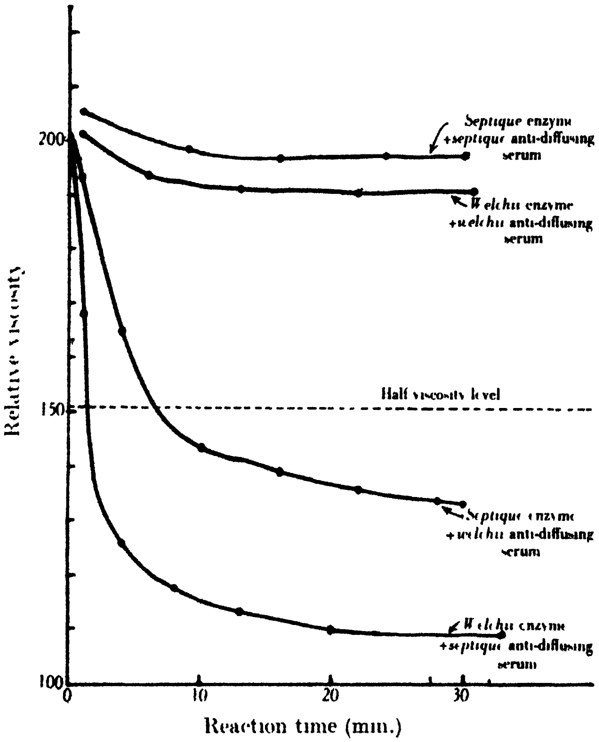


Fig. 2. The neutralization of hyaluronidases by anti-diffusing sera.

Table 4. *The influence of anti-diffusing sera on the liberation of N-acetylhexosamine*

Substrate: Vitreous humour.
Enzyme: Prepared from culture filtrate of *Cl. oedematis maligni* (*Vibrion septique*).

Serum	Liberation of N-acetylhexosamine (mg./ml.) in 18 hr. by falling dilutions of enzyme from *Vibrion septique*		
	Undiluted	1 : 10	1 : 100
Vibrion septique anti-diffusing serum	Nil	Nil	Nil
Cl. welchii anti-diffusing serum	1·0	0·1	Trace
Normal rabbit serum	1·0	0·1	Trace
Saline	1·0	0·1	Trace
Heated enzyme + saline + vitreous humour	Nil	—	—

inhibited the viscosity-reducing activity of the bacterial enzymes and the liberation of reducing substances by them. The influence on viscosity reduction is shown in Fig. 2 and on liberation of reducing substances in Table 4. Similar experiments on the inhibition of the activity of the testicular enzyme could not be undertaken since the purified enzyme from this source has never been obtained in an antigenic form.

Equal volumes of undiluted serum and the enzyme preparations were mixed and left at room temperature for 30 min. before addition to the substrate. In the observation on viscosity-reducing activity both *Cl. welchii* and *Vibrion septique* enzymes were set up with the homologous and heterologous antisera using a substrate of mucoprotein derived from umbilical cord. In the observation on the liberation of acetylhexosamine the enzyme from *Vibrion septique* was the only one used; it was set up against the homologous antiserum, against a *welchii* antiserum, normal rabbit serum and saline. The amount of acetylhexosamine liberated was estimated after 18 hr. incubation at 37°. It will be seen that in both experiments the inhibition of enzyme activity was complete and strictly specific; the heterologous antisera and normal serum exerted no effect. The diffusing activity of these enzymes in the skin occurs at a much higher dilution than that at which any *in vitro* viscosity-reducing activity can be demonstrated. It has already been shown [McClean, 1936] that these antisera can only completely inhibit a limited number of minimal diffusing doses of diffusing factor in the skin and this probably explains the failure of Meyer *et al.* [1940, 2] to demonstrate inhibition of diffusion with pneumococcal antiserum.

Influence of electrolytes on the reaction. Madinaveitia & Quibell [1940] employed a final concentration of 0·66 *M* NaCl and 0·166 *M* sodium citrate in their viscosimetric tests. For reasons given in the section describing our methods, we omitted the NaCl from our system, but retained the 0·166 *M* sodium citrate buffer. A comparison of estimations of the activities of several samples of both testicular and bacterial enzymes revealed a gross disparity between us and led to the investigation of the influence of electrolytes on this reaction.

The apparent viscosity reducing activities of a purified testicular enzyme and of purified enzymes derived from *Cl. welchii* and *Vibrion septique* were compared at concentrations of NaCl varying from nil up to *M*. The concentration of sodium citrate at 0·166 *M* was kept constant throughout the experiment. The influence of varying concentration on the initial viscosity of the substrate was controlled in all titrations of enzyme activity. The result of this experiment is shown in Fig. 3 in which the apparent strength of the enzyme is expressed in viscosity-reducing units plotted against NaCl concentration.

It will be seen that there is a marked retarding effect on enzyme activity as the salt concentration is increased above 0·3 *M*, but that below this level there is an apparent difference between the behaviours of testicular and bacterial enzymes; the testicular enzyme shows a rapid apparent fall in activity as the salt concentration is reduced to nil, whereas the activity of the bacterial enzymes appears to be maintained at a high level. Madinaveitia (personal communication) has shown that in salt concentrations below 0·1 *M* the direct relationship between enzyme concentration and reaction time on viscosity breaks down and that this lack of correlation is more marked for weak enzyme preparations. Since the method of estimating enzyme activity depends upon the employment of a dilution of the enzyme which will give a reaction time of 15–45 min. it is possible that considerable errors may develop in the assay of activity at very low salt concentrations. Thus it appeared that the difference between the behaviours of testicular and bacterial enzymes might be more apparent than real. Further-

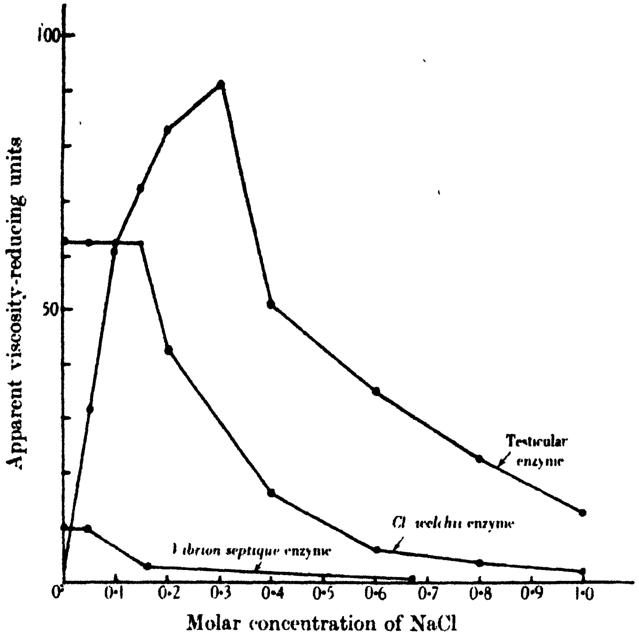


Fig. 3. The influence of NaCl concentration on the viscosity-reducing activity of testicular and bacterial enzymes.

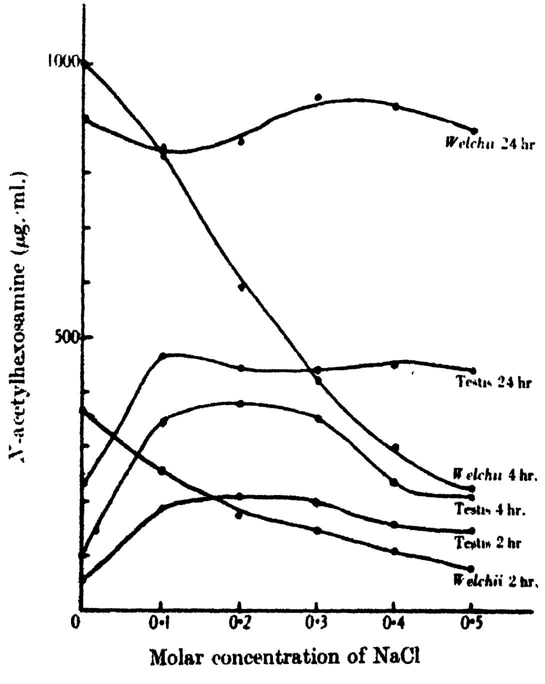


Fig. 4. The influence of NaCl concentration on the rate of liberation of *N*-acetylhexosamine by testicular and bacterial enzymes.

more, the purified bacterial enzymes were derived from culture filtrates and might contain small amounts of salts or other impurities sufficient to maintain a high level of activity. It should be stated, however, that both the testicular and bacterial enzymes had been exposed to prolonged dialysis during purification, were of considerable potency and were therefore used in dilutions which should have sufficed to eliminate the influence of impurities.

In order to obtain confirmatory evidence of the influence of salt concentration on the rate of the reaction, an experiment was performed to compare the rates of liberation of acetylhexosamine by testicular and *welchii* enzymes in the presence of NaCl concentrations ranging from nil to $0{\cdot}5M$. The amount of acetylhexosamine was estimated after 2, 4 and 20 hr. incubation at 37°. The result is shown in Fig. 4.

In this experiment ninety viscosity-reducing units of each enzyme were used; the enzymes were therefore much more concentrated than in the foregoing experiment and there is no question of direct proportionality between reaction time and enzyme concentration. It will be seen that after 2 and 4 hr. the maximum liberation of acetylhexosamine by the *welchii* enzyme has occurred at the lowest NaCl concentrations and that there is a steady fall as the salt is increased. The testicular enzyme, however, shows an increase in activity up to 0·2 or $0{\cdot}3\,M$ NaCl, similar to that which appears in the experiments on viscosity reduction; there is a fall in the yield as the salt concentration is further increased. There is no significant difference in the amount of acetylhexosamine liberated by either enzyme at any salt concentration after 24 hr.; the reaction is then presumably complete. Both activities appear to be similarly affected by variations in salt concentration and the difference between the viscosity-reducing activities of testicular and bacterial enzymes is confirmed in respect of the liberation of acetylhexosamine. These experiments show that the potency assigned to enzyme preparations will be profoundly affected by the NaCl concentration of the system and this has some bearing on the correlation between diffusing activity in the skin, which presumably occurs in isotonic salt, and *in vitro* hydrolysis of the substrate; this point will be considered later.

The part played by the different ions in the system was investigated. In order to determine the part played by the Na ion the concentration of sodium citrate at *p*H 4·6 was increased by amounts calculated to give a weight of Na equivalent to the various amounts of Na present as NaCl plus the constant amount of sodium citrate employed in the previous test. The activities of both testicular and bacterial enzymes at various sodium levels were estimated viscosimetrically in the normal way, allowance being made for the influences of sodium citrate concentrations on the initial viscosity of the system. The results showed that increase in Na concentration exaggerated the depressing effect on enzyme activity and almost eliminated the initial rise in the apparent activity of testicular enzyme which occurs in the presence of small concentrations of NaCl.

In order to vary the concentrations of the Cl ion in the presence of a constant amount of Na as citrate an experiment was set up in which the usual concentration of $M/6$ buffer was used in the presence of increasing concentrations of NH_4Cl. The result with both enzymes was in every way comparable with the action of Cl as NaCl.

A further experiment was performed on the influence of increasing concentrations of Na_2SO_4 in the presence of the constant amount of citrate buffer. The result obtained was the same as that with increasing amounts of sodium citrate.

These observations indicate that the accelerating action on both enzymes at low salt concentration is due to the Cl ion and that the Na ion exerts a depressing effect at similar concentrations. As the amount of salt is increased beyond a certain point both ions exert depressant influences. A preliminary account of these investigations together with those made by Madinaveitia has been published [Madinaveitia & McClean, 1940], and he has shown that the intensity of the apparent accelerating effect is in the order Cl > Br > I > SO_4 and the retarding effect at higher concentrations the opposite.

Relation of viscosity to concentration of substrate and the influence on enzymic activity. Madinaveitia & Quibell [1940] reported that the reaction constant and the half-life time of the viscous substrate are constant for a fixed amount of enzyme regardless of the concentration of the substrate. Robertson *et al.* [1940], on the other hand, found that the time taken for a fixed amount of enzyme to break down increasing amounts of substrate was proportional to the concentration of the latter. We have found that the reaction time of the enzyme is constant in concentrations of substrate varying from 0·66 to 6·6 mg./ml., thus confirming the findings of Madinaveitia and Quibell over a much greater range of viscous substrate than that of their experiment. In order to clear up this discrepancy we were led to investigate the relation of the viscosity to concentration of the substrate and the influence of NaCl on this relationship; we also wished to find out whether it is justifiable to use viscosity as a direct measurement of the concentration of mucous material or to assume that the half-viscosity level is that at which half of the substrate has been rendered non-viscous.

The viscosity of mucoprotein at concentrations increasing from 0·66 to 6·6 mg./ml., in the whole system as set up for enzyme titrations, was determined both in the absence of salt and in the presence of 0·66*M* NaCl. The results are shown in Fig. 5.

It will be seen that the viscosity increases at a much greater rate than the concentration of the substrate and that this effect is more marked as the concentration increases. Therefore the reduction of viscosity by half does not indicate that half the substrate has been rendered non-viscous. If we assume that when half-viscosity level is reached the substrate consists of a mixture of completely viscous and completely non-viscous material, the following conclusions can be drawn from these curves: as the concentration of the substrate is raised a gradually increasing amount but decreasing proportion of the total weight of substrate is rendered non-viscous when half-viscosity level is reached. Whether NaCl is present or not, the proportion of substrate rendered non-viscous at this level is approximately the same; therefore we think it is justifiable to assume that the half-viscosity level at different NaCl concentrations does represent a comparable decrease in the concentration of viscous material, but it is not true to say that the percentage decrease in the concentration is independent of the original concentration of mucoprotein. As already confirmed by us, however, it is an experimental fact that the reaction time of a sample of enzyme remains constant and is completely independent of the concentration of the substrate. Since the proportion of substrate destroyed by the enzyme is not constant in different concentrations of substrate this raises interesting questions as to how the constant reaction time is attained. It may be that a balance is established between increased rapidity in the formation of enzyme-substrate complex as the concentration is raised and slowing of molecular movement due to the increased viscosity of the system.

These observations on the relation between viscosity and concentration of substrate provide a possible explanation for the apparent discrepancy between Madinaveitia & Quibell's findings that the reaction time is constant regardless of the concentration of substrate and the report by Robertson *et al.*, that the rate of reaction is proportional to the concentration. The latter workers took their reactions to an end-point when either the viscosity was completely destroyed or the precipitability of the mucoprotein was abolished. From the curves in Fig. 5 it can be deduced that if the original substrate contains 6·6 mg./ml. then, when half-viscosity level is reached, only 1·45 mg./ml. will have been destroyed and 5·15 mg./ml. will remain. If, however, the original substrate only contains 0·66 mg./ml., then at half-viscosity level 0·30 mg./ml. will have been destroyed and only 0·32 mg./ml. remain. Therefore the latter reaction, at the lower initial concentration, will probably be carried to completion more quickly, although the time taken to reach half-viscosity level is the same with both samples of substrate.

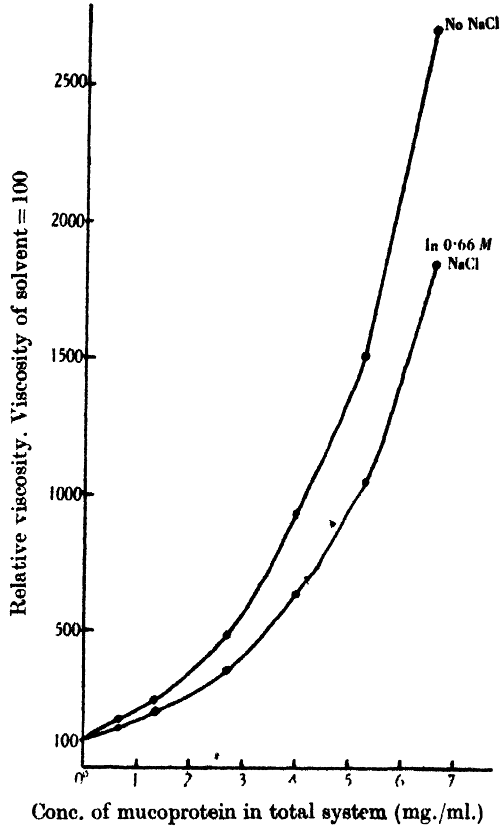


Fig. 5. The relation of viscosity to concentration of mucoprotein.

Relation of hyaluronidase activity to amylase and phosphatase, and to the action on other polysaccharides

Robertson *et al.* [1940] stated that mucinase causes a loss of viscosity of starch paste and that in some respects mucolytic and amylolytic activities are similar. They also stated that the first stage in the breakdown of mucin is catalysed by serum phosphatase. It seemed desirable, therefore, to investigate the amylolytic and phosphatase activities of our testicular and bacterial hyaluronidase preparations in order to determine whether there is any correlation between these activities.

Pastes were prepared from rice, maize, potato and wheat starches according to the method described by Waldschmidt-Leitz & Mayer [1935] except that the concentration of starch employed was slightly less than half that used by these authors. In the experiments with testicular and bacterial enzymes the proportions of enzyme and of sodium citrate buffer to the starch substrate were the same as those used in the determination of hyaluronidase activity with mucoprotein substrate.

Preliminary observations indicated that testicular enzyme did not reduce the viscosity of any of these starch pastes and that purified enzymes derived from *Cl. welchii* and *Vibrion septique* reduced the viscosity of both potato and wheat starch, but that the rate of the reaction bore no relation to their hyaluronidase activities.

An experiment, the results of which are shown in Table 5, was performed to compare the amylase and hyaluronidase activities of several bacterial and testicular preparations and to determine whether an increase in NaCl concentration exerts an effect on the amylase activity similar to that on the hyaluronidase.

Table 5. *A comparison of the amylase and hyaluronidase activities of various enzyme preparations*

Source of enzyme		NaCl concentration	Amylase activity. Viscosity reduction	Hyaluronidase activity. Viscosity-reducing units
V. septique	A	Nil	30 min. to reach H.V.L.	13
,,	B	Nil	35 min. to reach H.V.L.	3·5
,,	B	0·1 *M*	2 hr. 14 min. to reach H.V.L.	—
,,	B	0·2 *M*	2 hr. 50 min. to reach H.V.L.	—
,,	C	Nil	No activity	10
Cl. welchii	A	Nil	392–334 in 1 hr. 14 min. (H.V.L. = 246)	55
,,	B	Nil	354–340 in 1 hr. 35 min. (H.V.L. = 227) H.V.L. not reached in 21 hr.	120
Testis		Nil	No activity	2·5
,,		0·3 *M*	No activity	91

H.V.L. = Half-viscosity level.

Viscosity figures refer to relative viscosity. Buffer + heated enzyme = 100.

It will be seen that there is no correlation between the hyaluronidase and amylase activities of these preparations and that the addition of NaCl up to a final concentration of 0·3 *M* does not produce any amylolytic activity in the testicular enzyme, in striking contrast to its marked enhancing action on the hyaluronidase activity of the same specimen. The addition of NaCl produces a retarding effect on amylolytic activity of the *Vibrion septique* preparation similar to that produced on the hyaluronidase activity of bacterial enzymes.

When the starch pastes used were hydrolysed with $N/10$ H_2SO_4 a positive Benedict reaction was produced after boiling for 15 min. No Benedict reaction could be obtained even after 24 hr. incubation with the bacterial enzymes notwithstanding the substantial fall in viscosity that had occurred; the iodine reaction also remained positive. Takadiastase, which contains no hyaluronidase and does not diffuse in the skin, causes a rapid fall in the viscosity of starch paste with the liberation of reducing sugars.

We are indebted to Dr King of the Post-Graduate Medical School, London, for the phosphatase estimations on samples of hyaluronidase from various sources. The method employed has been described by King [1939]; disodium phenyl phosphate was employed as substrate for the phosphatase estimations and diphenyl phosphate for the estimations of phosphodiesterase.

Table 6. *A comparison between the hyaluronidase, phosphatase and phosphodiesterase activities of various enzyme preparations*

Source of enzyme	Hyaluronidase activity. Viscosity-reducing units	Primary phosphatase units		Phosphodiesterase units	
		*p*H 4·5	*p*H 10	*p*H 4·5	*p*H 10
Testis	10	3·0	1·8	Nil	3
Cl. welchii A	118	4·2	Nil	Nil	1
„ B	54	2·1	—	Nil	—
V. septique A	8	—	Nil	—	—
„ B	20	3·4	—	Nil	—
Viper venom A	10	—	6·4	—	Nil
„ B	3	2·1	—	Nil	—
S. typhi	Nil	25	Nil	Nil	Nil
Takadiastase (1%)	Nil	68	—	Nil	—

It will be seen that two samples of enzyme from *Cl. welchii* purified by different methods and of widely different hyaluronidase activities are included in the list and that samples of viper venom were tested in addition to the testicular and bacterial enzymes. A filtrate from a culture of *S. typhi* was included as an example of a bacterial filtrate containing no hyaluronidase and a solution of takadiastase as a control substance known to contain a phosphatase active at *p*H 4·6. These estimations indicate that, when the above-mentioned phosphoric esters are employed, there is no evidence of any correlation between the phosphatase or phosphodiesterase and the hyaluronidase contents of any of these samples; the phosphatase activities of all the samples were very slight. It appears that amylase and phosphatase play no part in the hyaluronidase activity of any of these enzyme preparations.

Neither the testicular nor bacterial enzymes exerted any action on the viscosity of gum acacia or that of the bacterial polysaccharide isolated from *Bact. dysenteriae* (Shiga) by Morgan & Partridge [1940].

Robertson *et al.* [1940] state that mucinase derived from *Cl. welchii* does not affect gastric mucin (human or swine), but Meyer *et al.* [1940, 1] report that whereas the enzymes from pneumococcus and streptococcus have no action on the neutral polysaccharide or mucoitin-sulphuric acid from pig gastric mucosa, the enzyme from *Cl. welchii* does hydrolyse these substrates. We tested the viscosity-reducing activity of hyaluronidase from testis, streptococci, *Vibrion septique* and *Cl. welchii* on a 1 % solution of commercial gastric mucin (granular mucin, type 1701 W. Wilson Laboratories), with the results presented in Table 7. In addition we performed an experiment similar to that to be described in the section on the part played by hyaluronidase in bacterial metabolism; *Cl. welchii* was cultivated in a basal medium alone and with the addition of glucose, potassium hyaluronate or gastric mucin. As will be seen in the second part of Table 7, the addition of glucose causes a slight increase and the addition of hyaluronate a considerable increase in the production of hyaluronidase. The inclusion of gastric mucin results in the production of less hyaluronidase than in the culture containing glucose, but the action of this filtrate on gastric mucin shows considerable enhancement.

Our results show no correlation between hyaluronidase activity and the action on gastric mucin. Whereas testicular and streptococcal enzymes of considerable hyaluronidase potency have no action on gastric mucin, a weak hyaluronidase from *Vibrion septique* caused a slight fall in the viscosity of gastric

Table 7. *The relationship between hyaluronidase and an enzyme which hydrolyses mucin from pig gastric mucosa*

	Source of enzymes	Hyaluronidase. Viscosity-reducing units. *p*H 4·6	Fall in viscosity of gastric mucin in 2 hr. (undiluted sample of enzyme). *p*H 7·0
I.	Testis extract (no NaCl)	2·5	No activity
	Testis extract (in 0·3 *M* NaCl)	90	No activity
	Streptococcus filtrate	330	No activity
	Vibrion septique filtrate	10	292–272 (7 %)
II.	*Cl. welchii* filtrate. Medium:		
	A. 3·5 % Evans peptone alone	15	268–256 (7 %)
	B. 3·5 % Evans peptone + 0·5 % glucose	180	268–237 (18 %)
	C. 3·5 % Evans peptone + 0·5 % potassium hyaluronate	590	268–246 (13 %)
	D. 3·5 % Evans peptone + 0·5 % gastric mucin	77	268–213 (33 %)

Note. No action on gastric mucin by any of these enzymes could be detected at *p*H 4·6.

mucin. *Cl. welchii* enzymes of widely different hyaluronidase activities possessed slight actions on gastric mucin which were not proportional to their activities on umbilical mucoprotein. The action of *Cl. welchii* on gastric mucin is, however, exalted by cultivation in the presence of this substance. Culture filtrates from *Vibrion septique* and *Cl. welchii* both liberate reducing substances from gastric mucin.

Relation of hyaluronidase in viper venom to its coagulant action on blood

Russell's viper venom which diffuses in the dermis also causes a rapid fall in the viscosity of mucoprotein with the liberation of reducing substances. This venom promotes the coagulation of haemophilic blood, but its mode of action has not been completely explained. Since the chemical constitution of heparin is closely allied to that of hyaluronic acid, it was thought that the hyaluronidase in the venom might destroy the heparin present in the circulating blood and thus accelerate coagulation. It was, therefore, of interest to determine whether this venom and the hyaluronidase from testis and *Cl. welchii* exert any destructive action on heparin and whether they accelerate the coagulation of blood.

Several experiments were performed in which plasma, to which sufficient heparin had been added to delay coagulation at 37° for 1–2 hr., was mixed with varying amounts of viper venom and with testicular and bacterial enzyme preparations. These observations were made with heparinized plasma alone and in the presence of 0·1 % lecithin which itself reduces the anticoagulant action of heparin. Control tests were made with the addition of varying amounts of brain pulp as a source of thrombokinase to the heparinized plasma; this pulp induced rapid coagulation.. No evidence was obtained from many experiments that viper venom, testicular or *Cl. welchii* enzymes reduce the anticoagulant action of heparin or that they act in a manner comparable with thrombokinase.

A further experiment was performed to determine whether viper venom, testicular or bacterial enzymes exert any direct destructive action on heparin in the absence of plasma. Heated and unheated samples of these enzymes were mixed with constant volumes of a heparin solution of known strength and the mixtures were incubated for 18 hr. at 37°. After incubation the enzymes were inactivated by boiling for 30 min. and, after cooling, a constant volume of blood was added to each tube. There was no reduction of clotting time in those tubes in which the heparin had been exposed to the various enzyme preparations; it can therefore be concluded that the heparin molecule was still intact and these observations confirm and extend the statement by Meyer *et al.* [1940, 1] that the enzymes obtained by them from pneumococci and *Cl. welchii* do not hydrolyse heparin. In view of the uniformly negative results of all our experiments they are not presented in detail, but we wish to record our thanks to Dr R. G. MacFarlane of the Wellcome Physiological Research Laboratories for supplies of lecithin emulsion and for much invaluable advice on the arrangement and interpretation of these experiments.

Artificial diffusing factors; influence on the viscosity of mucoprotein and diffusion in the dermis

Claude [1935] reported that azoproteins obtained by coupling diazobenzenesulphonic acid with serum, albumin, casein or gelatin diffuse in the skin in a manner comparable with testicular extract. Favilli [1940] reported that these azoproteins, diazobenzenesulphonic acid and its sodium salt reduce the viscosity of synovial fluid, but he encountered difficulties in deciding whether reducing substances are liberated as a result of this action. We have confirmed Favilli's observation that diazotized compounds reduce the viscosity of mucoprotein and that the fall in viscosity is slow compared with that produced by naturally occurring enzymes. The relative slowness of this reaction is of interest, since it is comparable with the rate of diffusion of these compounds in the skin; the greatest diffusion of naturally occurring enzymes takes place in the first 20 min. after injection, while that produced by diazotized compounds develops slowly, is not obvious for an hour or more and attains its maximum after 9 hr.

We have overcome the difficulties which arise in determining whether reducing substances are liberated. These difficulties are due to the coloured nature of the diazotized compounds, their own reducing power and the difficulty of removing soluble protein. After the azoprotein had been incubated with the mucoprotein substrate for 18 hr., attempts were made to remove the protein by precipitation with ammonium sulphate or with neutral lead acetate. Neither of these methods was satisfactory; treatment with ammonium sulphate resulted in a heavy precipitation when Ehrlich's reagent was added in the estimation of acetylhexosamine and, after treatment with neutral lead acetate it was impossible to remove the lead with H_2S in the usual manner. It was found, however, that if basic lead acetate (B.D.H.) is added to the mixture of azoprotein and substrate, to which sufficient NaOH has already been added to produce a deep red colour, the protein is precipitated and the residual lead can be removed with H_2S; the latter gas is removed by bubbling air through the mixture. The efficacy of this method was tested on samples to which a known quantity of acetylglucosamine had been added and also on mixtures of substrate with natural enzyme in which reducing substances had been liberated. Our observations were also controlled by tests on mixtures of the substrate with normal serum and with water and on the substrate alone. When acetylglucosamine is estimated in the usual manner by Morgan & Elson's method a yellow colour develops on the addition of Erhlich's reagent which alters the final colour slightly, but this can be compensated for by blending with the contents of known positive and negative tubes. Total reducing sugars can be estimated by the method of Hagedorn & Jensen after this lead treatment. Our observations indicate that after the viscosity of mucoprotein has been reduced by the action of diazotized compounds no increase of reducing substances can be detected; there is no evidence that diazotized compounds destroy reducing substances already present in the mixture.

As already explained in the section describing our methods, vitreous humour was replaced by mucoprotein from umbilical cord as substrate in our observations because of the spontaneous loss of viscosity by the former substance. Robertson *et al.* [1940] suggested that this loss of viscosity is due to traces of ascorbic acid and stated that the first stage in the breakdown of mucin is catalysed rapidly by 'ascorbic acid-peroxide'. It was of interest to investigate the nature of this reaction and to determine whether ascorbic acid and its oxidation products show any diffusing action in the skin.

Ascorbic acid was dissolved in freshly boiled distilled water at $M/5$ and $M/50$ concentrations. Samples of each concentration were taken, the first being treated with one molecular equivalent of H_2O_2, the second with two molecular equivalents and the third not treated. These specimens were tested for their action on the viscosity of the usual mucoprotein substrate buffered at pH 4·6. The fall in viscosity caused by all three specimens at $M/50$ concentration was extremely rapid, but it appeared that the sample containing one equivalent of H_2O_2 was slightly more active than the other two. The test was repeated with $M/500$ solutions, giving a final concentration of $M/3000$ in the system, and the result is shown in Fig. 6. It will be seen that the ascorbic acid and its oxidation products all cause a rapid fall in viscosity, but that the specimen with one equivalent is slightly more active than the other two, half-viscosity level being reached in less than 15 min.

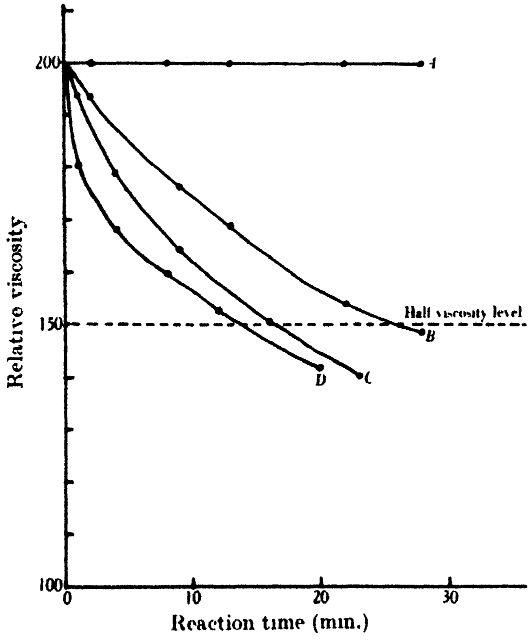


Fig. 6. The reduction of viscosity by ascorbic acid derivatives. A = control, water only. $B = M/3000$ ascorbic acid + two equivalents of H_2O_2. $C = M/3000$ ascorbic acid alone. $D = M/3000$ ascorbic acid + one equivalent of H_2O_2.

In order to test the diffusing activity of these preparations in the dermis it was necessary to buffer them at pH 7·0 before injection. The results of these diffusion tests are shown in Fig. 7. The diffusion of all three preparations in the skin is marked but develops slowly, attaining a maximum after 3 hr., and is of the same type as the spread due to diazotized compounds; a curve illustrating the typical rapid diffusion of testicular enzyme is included in the figure for comparison. The preparation with one equivalent of H_2O_2 spread over a greater area than either of the others.

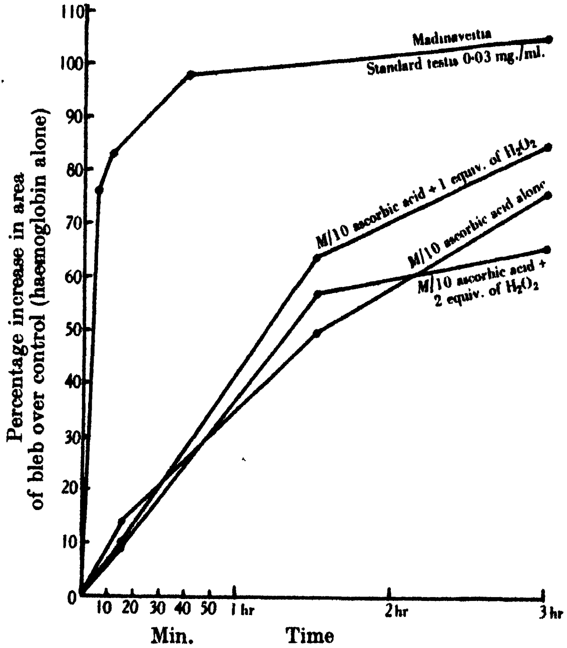


Fig. 7. The diffusion of ascorbic acid derivatives compared with that of testis diffusing factor.

It was thought that the action of ascorbic acid and its products on the viscosity of mucoprotein might be reversible. Samples of the substrate were taken after 40 min. and 18 hr. exposure to the dehydroascorbic acid, together with controls of substrate with water, and these were dialysed for 48 hr. The viscosity of the dialysed material was measured, but no evidence was obtained of any recovery of viscosity.

Notwithstanding the extremely rapid fall in viscosity of the substrate, no detectable acetylhexosamine is liberated by ascorbic acid or its derivatives; in this respect the action resembles that of the diazotized compounds. The addition of enzyme obtained from *Cl. welchii* to substrate which has already been exposed to ascorbic acid oxidation products results in the liberation of the normal amount of acetylhexosamine.

The characteristics of the action of these preparations on mucoprotein and their diffusion in the skin differ in several respects from that of naturally occurring hyaluronidase. The failure to liberate acetylhexosamine and the slow diffusion in the skin has already been noted. In addition we observed that the reaction time measured viscosimetrically is not proportional to the concentration of ascorbic acid or its products that are present. The increase in reaction time as the dilution is increased from *M*/5 to *M*/500 is very small. The reaction time is not independent of the concentration of substrate as it is with the naturally occurring enzymes; increase in this concentration causes a marked lengthening of the reaction time. The dermal diffusion of naturally occurring enzymes is evident at a much higher dilution than that at which reduction of viscosity of mucoprotein can be detected; on the other hand, the viscosity-reducing activity of ascorbic acid and its derivatives can be detected in dilutions much greater than those necessary to demonstrate dermal diffusion.

An accidental observation in the course of some control tests revealed the fact that 0·0066 M thiolacetic acid reduces the viscosity of mucoprotein and that this reduction passes the half-viscosity level during 18 hr. incubation. This led us to investigate whether other reducing substances, such as H_2S, quinol, pyrogallol, glucose, sodium sulphite and 'metol', had similar actions and whether they showed any diffusing action in the skin. Tests were made to exclude the possibility that these substances activated an enzyme already present in the substrate and to determine whether reducing substances were liberated as a result of the action on viscosity. All the above substances, with the exception of glucose, cause a similar reduction of viscosity of the substrate and none of them liberate reducing substances; the viscosity of heated substrate is reduced, which excludes the possibility that an enzyme already present is activated by these agents. Several of these substances produce definite diffusion in the skin which is of the slow type similar to that caused by diazotized compounds and ascorbic acid. This activity and its relation to the reduction potential of the various substances is being investigated and the results will be described in a later communication; it is hoped that it may help to elucidate the mode of action of diazotized compounds and ascorbic acid.

The influence of hyaluronidase on bacterial metabolism

Preliminary experiments have been made to determine whether bacteria which produce hyaluronidase can use hyaluronic acid as a metabolite if it is included in the culture medium and what influence the presence of this substance has upon the production of the enzyme by the organism.

A rapidly growing 2 hr. culture of *Cl. welchii* was washed in saline to remove any hyaluronidase present in the culture fluid; the washed suspension was used as inoculum for tubes containing the following four media:

(*a*) A basal medium of 3·5 % Evans peptone alone.

(*b*) 3·5 % Evans peptone + 0·5 % glucose.

(*c*) 3·5 % Evans peptone + 0·5 % potassium hyaluronate.

(*d*) The same composition as (*c*) with the addition of 1 unit of hyaluronidase to start the enzymic action on the hyaluronate.

Anaerobiosis was maintained in these tubes by the addition of 1 % by vol. of a 2 M thiolacetic acid in N HCl: the pH was corrected by neutralization with NaOH. The cultures were incubated for 18 hr.; the amount of bacterial multiplication was estimated by comparison with standard opacity tubes; the hyaluronidase and spreading activities of the culture fluids were titrated in the usual way. The result of this experiment is recorded in Table 8.

It will be seen that the addition of glucose to the medium results in an eightfold increase in bacterial multiplication and an approximately fivefold increase in hyaluronidase production. The substitution of potassium hyaluronate for glucose does not significantly increase multiplication of the organism, but the concentration of hyaluronidase shows a dramatic ninefold increase as compared with the medium containing glucose; this is accompanied by a considerable increase of spreading activity in the skin. The medium containing 1 unit of hyaluronidase shows a further slight increase of hyaluronidase which may be significant. This experiment, which has been repeated with consistent results, shows that *Cl. welchii* can use a salt of hyaluronic acid as a metabolite and that the inclusion of the specific substrate in the medium induces a greatly enhanced production of hyaluronidase.

Table 8. *Cultivation of* Cl. welchii *in various media*

Medium	Bacterial multiplication. Opacity tube number	Hyaluronidase. Viscosity-reducing units	Dermal diffusion. Highest dilution producing diffusion
3·5 % Evans peptone alone	1	13	10^{-5}
3·5 % Evans peptone + 0·5 % glucose	8	70	10^{-7}
3·5 % Evans peptone + 0·5 % potassium hyaluronate	8	1340	10^{-9}
3·5 % Evans peptone + 0·5 % potassium hyaluronate + 1 unit *welchii* hyaluronidase	8	1660	10^{-9}

Infections by *Cl. welchii* are characterized by extremely rapid spread through the tissues and marked generalized toxaemia. The rapid adaptation of this organism in the presence of a salt of hyaluronic acid in the culture medium is of considerable interest in this connexion. It is known that there is a mucinous interfibrillar structure in the tissues, and recently Claude [1940] and Chain & Duthie [1940] have described the extraction from the skin of a substance indistinguishable from hyaluronic acid which is destroyed by hyaluronidase. We have also found that reducing substances are liberated by the action of this enzyme on crude extracts from the skin. Therefore, it appears likely that as soon as *Cl. welchii* start to proliferate in the tissues the hyaluronic acid there is attacked by means of this enzyme. Thus the organism is able to use this metabolite and, at the same time, by destroying the viscosity of this material to increase the permeability of the tissues and render more of the specific substrate accessible. In addition, the presence of hyaluronic acid causes the organism to produce greatly increased amounts of the enzyme. In this way a vicious circle is set up that promotes the rapid extension of the infection.

Further investigations on this adaptive enzyme effect with other strains of *Cl. welchii* are in progress and similar observations on strains of streptococcus and staphylococcus which do and those which do not produce the diffusing factor are being made. An attempt is being made to correlate this phenomenon with the relative virulence or local invasiveness of different strains of these organisms, but results of these observations will be reported in detail elsewhere.

Neither sulphanilamide nor sulphapyridine (M and B 693) inhibit the *in vitro* production of hyaluronidase by *Cl. welchii* when these substances are introduced into the culture medium. Neither of the substances have any direct action on the viscosity of the mucoprotein substrate, nor do they inhibit the *in vitro* activity of the enzyme.

Correlation between hyaluronidase activity and diffusion in the dermis

The close association between hyaluronidase activity and diffusion in the dermis that is obtained with tissue extracts, various bacterial extracts and snake venoms presents a strong *prima facie* case for assuming that the same enzyme is responsible for both phenomena. This assumption is further supported by the isolation of a substance from the skin [Claude, 1940; Chain & Duthie, 1940] which is indistinguishable from hyaluronic acid, and the viscosity of which is reduced by hyaluronidase. We have ourselves observed that enzyme preparations cause the liberation of reducing substances from a crude extract of skin. The fact that diazotized compounds, ascorbic acid and its oxidation products and other artificially prepared substances which reduce the viscosity of the substrate also diffuse in the skin, though in a slower manner than enzymes, adds support to the belief that both diffusion and viscosity reduction have an identical cause. Recently, however, Madinaveitia *et al.* [1940] have published a warning that it is not justifiable to assay skin-diffusing factors by merely measuring their hyaluronidase activity. Using the method of Bacharach *et al.* [1940], these authors failed to obtain parallel figures for the titrations of diffusing factor potency and hyaluronidase activity measured viscosimetrically and they suggest that it may be possible to separate diffusing factors from hyaluronidase. While the validity and relative accuracy of various methods of assaying diffusing factors is under investigation it may be suggested that conclusions based upon the lack of correspondence between titrations of diffusing factor and of hyaluronidase should be treated with caution. The environmental conditions of the test in the skin and that in the viscosimeter differ profoundly and may exert differential effects upon samples of enzymes from different sources or those from the same source purified by different methods. Viscosimetric tests were carried out by Madinaveitia & Quibell [1940] in $0{\cdot}66M$ NaCl and at *p*H 4·6, whereas skin diffusion presumably takes place in isotonic salt at or about neutrality. The marked influence of salt concentration and *p*H on the *in vitro* activity of the enzymes has already been demonstrated; it has also been shown that testicular and bacterial enzymes differ in their responses to changing salt concentration. There is also evidence that reducing substances affect the viscosity of the substrate and the permeability of the tissues; little is known at present about the oxidation-reduction potential of the *in vitro* enzyme system or of the tissues into which these factors are injected. Investigations on this point are in progress. In addition, there are many possible factors concerned in the response of an animal to these substances which it is not possible to control adequately at present. In the existing state of knowledge it is certainly unjustifiable to assay skin-diffusing factors in terms of hyaluronidase activity but, in view of the considerations set out above, it does not follow that the two activities are due to separate agents.

Diffusion in the skin is caused by enzyme preparations that are too weak to be titrated viscosimetrically and those of moderate hyaluronidase content produce significant diffusion at high dilution. The skin test is therefore very much more delicate though much less accurate than viscosimetric assay. In the section describing our methods, we have explained why, for the purposes of this work, we preferred to continue testing for diffusing activity by determining the minimal diffusing dose rather than by the methods devised by Madinaveitia or by Bacharach *et al.* When the limiting diffusing dilution is determined it is not claimed that differences of less than tenfold dilutions can be distinguished, but it is possible to determine whether samples assayed by this method fall into the same order of activity as they do when titrated viscosimetrically. Table 9 contains the result of such a comparison.

It will be seen that, broadly speaking, the enzyme samples fall into the same order of activity by both tests. There is slight overlapping of those samples showing limiting diffusion at dilutions of 10^{-4} and 10^{-5}, but otherwise there is as good agreement as could be expected when the limitations of the animal test are taken into consideration. Further work is required to determine how far the diffusion test of Bacharach *et al.* will give results in agreement with our test, and these comparative observations are in progress. In the meantime the results presented in this table support the belief that diffusion in the skin may be due to hyaluronidase activity. This conclusion is further supported by the work of Chain & Duthie [1940] which has just appeared; these authors find good quantitative agreement between hyaluronidase activity determined viscosimetrically and spreading activity in the skin with various testicular extracts and with leech and bee venoms.

Table 9. *Correlation between diffusion in the dermis and viscosity-reducing activity*

Source of enzyme	Highest dilution producing diffusion in the dermis	Viscosity-reducing units (in 0·146 *M* NaCl)
Cl. welchii filtrate 1 *a*	10^{-3}	6·5
Cl. welchii filtrate 1	10^{-4}	13
Cl. welchii (purified by method of Morgan & McClean [1932])	10^{-4}	25
Cl. welchii (purified by method of Robertson *et al.* [1940])	10^{-5}	18
Cl. welchii (purified by method of Morgan & McClean)	10^{-5}	25
Cl. welchii filtrate 2 *a*	10^{-5}	29
Testis, Glaxo standard, 1 mg./ml.	10^{-5}	36
Testis (purified by method of Morgan & McClean)	10^{-5}	70
Cl. welchii filtrate 2	10^{-6}	70
Cl. welchii filtrate 3 *a*	10^{-7}	920
Cl. welchii filtrate 3	10^{-8}	1340
Cl. welchii filtrate 4	10^{-8}	1660

SUMMARY

1. The association between hyaluronidase activity and diffusion in the tissues of testicular extracts, certain bacterial filtrates and snake venom is described.

2. The influence of *p*H on the hyaluronidase activity of these substances has been investigated.

3. The liberation of reducing substances by the enzymes has been studied. Quantitative comparisons of viscosity-reducing activity and the time relation of both reactions suggest that two separate mechanisms may be involved.

4. The reduction of viscosity and liberation of reducing substances by bacterial enzymes can be completely neutralized by appropriate antisera which also inhibit diffusion in the skin. This neutralization is strictly specific.

5. A study of the influence of the concentration of electrolytes on the reduction of viscosity and liberation of reducing substances by testicular and bacterial enzymes reveals that the relative potency assigned to enzyme samples from different sources is profoundly modified by the salt concentration of the system. Testicular enzymes appear to differ in their behaviour from bacterial enzymes as the salt concentration is reduced.

6. The relation between viscosity and concentration of substrate has been investigated. The viscosity is not proportional to the concentration. The bearing of this observation on estimation of enzyme activity is discussed.

7. There is no correlation between the hyaluronidase activities of enzymes from various sources and their amylase or phosphatase contents, nor with their actions on gastric mucin or several other polysaccharides.

8. The hyaluronidase of Russell's viper venom ('Stypven') is not associated with any action on heparin. Neither this venom nor testicular or bacterial enzymes have any direct action on heparin, nor do they promote the coagulation of heparinized plasma.

9. Diazotized compounds are known to diffuse in the dermis; the observation that they also reduce the viscosity of mucoprotein has been confirmed. These compounds do not liberate reducing substances.

10. Ascorbic acid and its oxidation products cause a rapid fall in the viscosity of mucoprotein without the liberation of reducing substances. They also diffuse in the skin in a manner similar to diazotized compounds.

11. Various reducing agents also cause a fall in viscosity of the substrate and diffuse in the skin. The action of these artificially prepared compounds is compared with that of naturally occurring enzymes. Further investigations on the activity of artificial compounds are in progress.

12. Preliminary experiments on the influence of the inclusion of potassium hyaluronate in the culture medium of *Cl. welchii* are reported. A large increase in the yield of hyaluronidase results. The significance of this increase in relation to the proliferation of these organisms in the tissues is discussed. Further observations with this and other organisms that produce hyaluronidase are in progress.

13. Sulphanilamide and sulphapyridine do not inhibit production of this enzyme by *Cl. welchii* nor do they inhibit the *in vitro* activity of the enzyme.

14. The question whether diffusion in the tissues is due to hyaluronidase is discussed. The correlation between tests for the two activities is considered. Our experimental results support the belief that diffusion in the skin is due to hyaluronidase activity.

We wish to express our appreciation of the interest and advice of Dr B. C. J. G. Knight throughout this investigation.

REFERENCES

Bacharach, Chance & Middleton (1940). *Biochem. J.* **34**, 1464.
Boyland & McClean (1935). *J. Path. Bact.* **41**, 553.
Chain & Duthie (1939). *Nature, Lond.*, **144**, 977.
—— —— (1940). *Brit. J. exp. Path.* **21**, 324.
Claude (1935). *J. exp. Med.* **62**, 229.
—— (1937). *J. exp. Med.* **66**, 353.
—— (1940). *Proc. Soc. exp. Biol., N.Y.*, **43**, 684.
—— & Duran-Reynals (1937). *J. exp. Med.* **65**, 661.
Duran-Reynals (1933). *J. exp. Med.* **58**, 161.
—— (1939). *J. exp. Med.* **69**, 69.
—— & Stewart (1931). *Amer. J. Cancer*, **15**, 2790.
Favilli (1936). *Sperimtale*, **90**, 304.
—— (1940). *Nature, Lond.*, **145**, 866.
Hoffman & Duran-Reynals (1931). *J. exp. Med.* **53**, 387.
King (1939). *Biochem. J.* **33**, 1185.
Madinaveitia (1938). *Biochem. J.* **32**, 1806.
—— (1939, 1). *Biochem. J.* **33**, 347.
—— (1939, 2). *Biochem. J.* **33**, 1470.
—— & McClean (1940). *Chem. & Ind.* **59**, 850.
—— & Quibell (1940). *Biochem. J.* **34**, 625.
—— Todd, Bacharach & Chance (1940). *Nature, Lond.*, **146**, 197.
Manozzi-Torini (1939). *Arch. Sci. Biol.* **25**, 473.
McClean (1930). *J. Path. Bact.* **33**, 1045.
—— (1931). *J. Path. Bact.* **34**, 459.
—— (1936). *J. Path. Bact.* **42**, 477.
McClean & Hale (1940, 1). *Nature, Lond.*, **145**, 866.
—— —— (1940, 2). *Chem. & Ind.* **59**, 347.
Meyer & Chaffee (1940). *Proc. Soc. exp. Biol., N.Y.*, **43**, 487.
—— Hobby, Chaffee & Dawson (1940, 1). *J. exp. Med.* **71**, 137.
—— —— —— —— (1940, 2). *Proc. Soc. exp. Biol., N.Y.*, **44**, 294.
—— & Palmer (1936). *J. biol. Chem.* **114**, 689.
Morgan & Elson (1934). *Biochem. J.* **28**, 988.
—— & McClean (1932). *J. Soc. Chem. Ind.* **51**, 912.
—— & Partridge (1940). *Biochem. J.* **34**, 169.
Robertson, Ropes & Bauer (1940). *J. biol. Chem.* **133**, 261.
Waldschmidt-Leitz & Meyer (1935). *Hoppe-Seyl. Z.* **236**, 168.

18. METABOLISM OF POLYCYCLIC COMPOUNDS

4. PRODUCTION OF A DIHYDROXY-1:2:5:6-DIBENZANTHRACENE FROM 1:2:5:6-DIBENZANTHRACENE

By ERIC BOYLAND, ALFRED AARON LEVI,[1]
ELINOR HUNTSMAN MAWSON[1] AND EDNA ROE[2]

From the Chester Beatty Research Institute, The Royal Cancer Hospital (Free), London, S.W. 3

(*Received 31 December 1940*)

When anthracene is fed to rats and rabbits different stereoisomeric forms of 1:2-dihydroxy-1:2-dihydroanthracene and their glycuronic acid derivatives are excreted in the urine [Boyland & Levi, 1935; 1936]. Extension of the work to the metabolism of the carcinogenic compound 1:2:5:6-dibenzanthracene in rabbits has led to the isolation of a phenol, dihydroxy-1:2:5:6-dibenzanthracene by Boyland & Levi [1938]. The same compound was later obtained by Dobriner *et al.* [1939]. No positive evidence of the presence of hydrogenated derivatives with asymmetric carbon atoms corresponding to those produced from anthracene has been found, although such compounds might be excreted and later decomposed to give the phenol.

Experimental

Rabbits were fed on the following diet:

Wholemeal flour	35 parts
Wheat germ	30 ,,
Dried milk	10 ,,
Lard containing 0·8 % of 1:2:5:6-dibenzanthracene	20 ,,
Cod liver oil	2 ,,
NaCl	2 ,,
Dried yeast	1 part

The rabbits which were maintained on this diet for many months died generally with cirrhosis of the liver, similar to that described as resulting from injection of 1:2:5:6-dibenzanthracene into rabbits [Polson, 1936; Claude, 1937; Burrows & Boyland, 1938]. In some experiments in which the same 1:2:5:6-dibenzanthracene diet was fed to rats no cirrhosis was produced but the animals died in 6–10 weeks. With both species proteinuria frequently developed during the experiments.

The animals were kept in metabolism cages and the urine was collected daily, filtered through kieselguhr and stored at 0–5°. The urine was fluorescent and the isolation of products was followed by continuous examination of extracts in ultraviolet light.

Examination of sulphur compounds

The partition of the sulphur present in the urine was determined as described by Folin [1905] and the results obtained with two rabbits to which 1:2:5:6-dibenzanthracene was administered are given in Table 1. The significance of the results was calculated by the method of '*t*' [Fisher, 1936]. In both cases there is an increase in the proportion of ethereal sulphate present, indicating that the dibenzanthracene may be partly excreted as a sulphuric ester of a hydroxy derivative.

---

[1] Halley Stewart Fellow.

[2] International Cancer Research Foundation Scholar.

In one case (rabbit B) there was a considerable increase in the amount of neutral sulphur particularly after the rabbit had been on the dibenzanthracene

Table 1. *The effect of administration of 1:2:5:6-dibenzanthracene to rabbits on the sulphur partition in the urine*

Rabbit A. 7 days on normal diet, then on diet containing 1:2:5:6-dibenzanthracene

Day	Percentage of total sulphur		
	Inorganic sulphate	Ethereal sulphate	Neutral sulphur
1	51·8	6·2	42·0
2	49·5	5·5	45·0
3	44·7	7·0	48·3
6	41·9	2·6	55·5
7	42·8	3·4	53·8
13	63·5	7·8	29·7
14	50·5	3·2	46·3
15	44·5	9·1	46·4
27	53·0	11·0	36·0
35	53·8	13·2	33·0
57	34·7	14·6	50·7
63	50·7	7·6	41·7
78	44·1	3·8	52·1
Mean on normal diet		4·9	48·9
Mean on dibenzanthracene diet		8·8	42·1
Probability '*P*' that difference in means is real		0·05	0·2

Rabbit B. 40 days on normal diet, then on diet containing 1:2:5:6-dibenzanthracene

Day	Percentage of total sulphur		
	Inorganic sulphate	Ethereal sulphate	Neutral sulphur
1	71·8	4·8	23·4
4	74·7	5·6	19·7
8	68·3	11·8	19·9
11	69·9	5·6	24·5
16	79·6	6·0	14·4
40	69·5	7·5	23·0
42	60·1	10·2	29·7
46	66·5	5·8	27·7
47	5·8	11·2	30·4
49	74·5	4·5	21·0
53	60·5	6·2	33·3
103	28·0	8·0	64·0
109	31·6	8·5	54·5
118	37·7	8·1	54·2
Mean on normal diet		6·9	20·6
Mean on dibenzanthracene diet		8·5	37·2
Probability '*P*' that difference in means is real		0·2	0·02

Table 2. *The effect of administration of 3:4:5:6-dibenzcarbazole to rabbits on the sulphur partition in the urine*

Rabbit C. 13 days on normal diet, then on diet containing 0·16% 3:4:5:6-dibenzcarbazole

Day	Percentage of total sulphur		
	Inorganic sulphate	Ethereal sulphate	Neutral sulphur
1	58·0	4·7	37·3
2	53·7	6·6	39·3
6	47·4	5·8	46·8
7	40·4	6·1	53·5
13	53·1	7·8	39·1
14	34·9	12·8	52·3
15	19·8	12·8	67·4
20	46·7	11·6	41·7
26	28·4	12·1	59·5
33	30·9	17·7	51·4
42	47·1	8·7	44·2
61	31·4	26·8	41·8
74	32·1	12·9	55·0
Mean on normal diet		6·2	43·2
Mean on dibenzcarbazole diet		12·8	51·7
Probability '*P*' that increase is real		0·05	0·05

diet for 50 days. It seems probable that this increase in neutral sulphur is due to proteinuria following hepatic and renal injury. No mercapturic acid was isolated whereas if the increased neutral sulphur had been due to dibenzanthracene mercapturic acid, large amounts of this compound would have been present and its isolation should not have presented difficulties.

Some similar results with the carcinogenic 3:4:5:6-dibenzcarbazole are given in Table 2. Again there is a marked increase in the amount of ethereal sulphate and a small but definite increase in the neutral sulphur fraction. Other chemical investigations indicated that 3:4:5:6-dibenzcarbazole is excreted as a fluorescent hydroxy derivative. It is not possible to say whether in this case the increase in neutral sulphur is due to mercapturic acid or excreted protein, but rabbits to which 3:4:5:6-dibenzcarbazole was administered developed proteinuria.

Other determinations were made on the collected urine of 12 rats on normal diets and diets containing carcinogenic compounds. On adding 1:2:5:6-dibenzanthracene to the diet the ethereal sulphate rose slightly, from 8·1 to 9·6 %, and the neutral sulphur from 24·1 to 26·7 % of the total sulphur in the urine. The addition of 3:4:5:6-dibenzcarbazole to the rats' diet similarly increased the ethereal sulphate from 11·3 to 14·7 % and the neutral sulphur from 31·6 to 34·6 % of the total urine sulphur. These experiments indicate that these two carcinogenic substances are also excreted as sulphuric esters in rat's urine.

Isolation of dihydroxy-1:2:5:6-dibenzanthracene

This substance was obtained from the urine in two ways:

(*a*) The dried and powdered kieselguhr and precipitate were extracted in a Soxhlet apparatus with ether for some hours. The fluorescent ether extract was washed repeatedly with very dilute Na_2CO_3 solution until no further acidic material was present. This process removed much acid, the main part of which appeared to consist of stearic acid; M.P. after two crystallizations from aqueous alcohol 60–61°, equiv. wt. 280, 288 (stearic acid equivalent weight 284, M.P. 62·2°).

The residual ether was then extracted two or three times with dilute NaOH. The acidified extract gave a yellow flocculent precipitate of a phenol, which was collected and dried and recrystallized from acetic acid.

(*b*) The filtered urine in 2 l. lots was acidified, shaken with butyl alcohol in a large funnel, and allowed to stand for 12 hr. The emulsion remaining in the butyl alcohol layer was largely removed by filtration through kieselguhr. The butyl alcohol was then mixed with one volume of light petroleum, washed with water, and extracted with several small portions of dilute NaOH until the extracts were only faintly coloured. The combined alkaline extracts were washed with ether to remove dissolved butyl alcohol, first made acid with HCl, then alkaline with Na_2CO_3, and the phenolic material extracted with ether. It was re-extracted with NaOH solution, precipitated with HCl, filtered, dried and recrystallized from acetic acid. It was identical with that obtained from the kieselguhr. From the two sources about 10 mg. were isolated from each litre of urine.

Dihydroxy-1:2:5:6-dibenzanthracene crystallized in yellow needles which darken on exposure to light; M.P. 340–350° with decomposition; soluble in alcohol, acetone and ether to give violet-fluorescent solutions; almost insoluble in $CHCl_3$ and benzene. It was not extracted from ethereal solution by Na_2CO_3 solution. The solution in NaOH was yellow with a bright green fluorescence and was fairly stable in air. The aqueous and alcoholic solutions gave no reaction with $FeCl_3$. A 1 % solution of the substance in dioxane ($c = 1$ %) showed no optical activity. Analysis (Schoeller), found: C, 84·2 %; H, 5·6 %. $C_{22}H_{14}O_2$ requires: C, 85·2 %; H, 5·5 %.

This phenol could be obtained by alkaline hydrolysis of the purified diacetyl derivative and was more conveniently purified and obtained in better yield in this way.

Diacetoxy-1:2:5:6-dibenzanthracene. The dihydroxy-1:2:5:6-dibenzanthracene obtained from urine was dissolved in one volume of pyridine and the solution boiled with ten volumes acetic anhydride for 5 min. The solution was hydrolysed with water and the product crystallized from acetic acid in colourless plates, M.P. 291°. It was slightly soluble in ethyl alcohol and the solution had a blue-violet fluorescence.

Analysis (Weiler), found: C, 78·0 %; H, 4·5 %. $C_{26}H_{18}O_4$ requires: C, 78·1 %; H, 4·6 %.

Diacetoxy-1:2:5:6-dibenzanthraquinone. Diacetoxy-1:2:5:6-dibenzanthracene on warming with CrO_3 in acetic acid gave a quinone which crystallized from hot acetic acid in yellow needles, M.P. 294–296°. It was soluble in alcohol and dissolved slowly in alkaline $Na_2S_2O_4$ to give a clear orange solution. 1:2:5:6-Dibenz-9:10-anthraquinone gives a red solution in alkaline $Na_2S_2O_4$. Analysis (Weiler), found: C, 78·3 %; H, 4·0 %. $C_{20}H_{16}O_6$ requires: C, 78·6 %; H, 3·8 %.

Dimethoxy-1:2:5:6-dibenzanthracene. Dihydroxy-1:2:5:6-dibenzanthracene treated with dimethyl sulphate in NaOH solution gave a methyl ether which crystallized from hot acetic acid in pale brown needles, M.P. 244–245°. It gave violet-fluorescent solutions in acetone and alcohol. Analysis (Schoeller), found: C, 85·0 %; H, 5·7 %. $C_{24}H_{18}O_2$ requires: C, 85·3 %; H, 5·3 %.

Dimethoxy-1:2:5:6-dibenzanthraquinone. Oxidation of the dimethyl ether with CrO_3 in acetic acid gave poor yields of the quinone, which crystallized from ethyl alcohol in red plates, M.P. 264°. Analysis (Schoeller), found: C, 77·2 %; H, 4·40 %. $C_{24}H_{16}O_4$ requires: C, 78·0 %; H, 4·32 %. The solution in NaOH with $Na_2S_2O_4$ was clear yellow.

Dihydroxy-1:2:5:6-dibenzanthraquinone. The diacetyl derivative of the quinone when warmed with 2*N* NaOH gave a deep blue solution from which a nearly black Na salt separated on standing. The blue solution turned orange on being warmed with Zn dust or $Na_2S_2O_4$.

The hydroxyquinone was prepared by warming the diacetyl derivative with alcoholic alkali for a few minutes. The deep blue solution was poured into dilute acid, when a red gelatinous precipitate separated. This was collected, dried and recrystallized from glacial acetic acid, when it formed deep red needles, M.P. 350°. Analysis (Schoeller), found: C, 77·0 %; H, 3·8 %. $C_{22}H_{12}O_4$ requires: C, 77·7 %; H, 3·5 %.

On heating with boracetic anhydride in acetic anhydride the dihydroxyquinone did not form a boroacetate, but the original quinone acetate was recovered. The substance was readily methylated with dimethyl sulphate in NaOH. Its blue alkaline solution was changed to green by the addition of diazotized sulphanilic acid.

The quinone (100 mg.) was oxidized with acid $KMnO_4$ under conditions identical with those described for the oxidation of 1:2:5:6-dibenzanthraquinone to anthraquinone tetracarboxylic acid [Cook, 1931]. No anthraquinone tetracarboxylic acid could be isolated, whereas had the hydroxyl groups been situated in the side rings this product should have been obtained.

Absorption spectra

The absorption spectra were measured by means of a Hilger medium quartz spectrograph and a Spekker photometer [cf. Mayneord & Roe, 1935]. Alcohol was used as solvent throughout. 1:5-Dihydroxyanthraquinone was sparingly soluble in alcohol and gave varying results when the suspension was warmed to hasten solution. The solution finally used was obtained by shaking for some hours in the dark.

A comparison of the spectrum of the phenol (dihydroxy-1:2:5:6-dibenzanthracene, Fig. 1) with that of 1:2:5:6-dibenzanthracene [Mayneord & Roe, 1935] indicates clearly that it is a 1:2:5:6-dibenzanthracene derivative. The spectrum is shifted 50–100 Å. towards the visible compared with 1:2:5:6-dibenzanthracene, its absorption intensity is diminished, and its long-wave band system is simpler in form.

Substitution by a methyl group in a side-ring of 1:2:5:6-dibenzanthracene, e.g. 2'- or 3'-methyl-1:2:5.6-dibenzanthracene, has very little effect on the absorption spectrum. The resulting spectrum shows slightly lower band-intensities and 2–20 Å. band-shifts towards the visible compared with that of 1:2:5:6-dibenzanthracene [E. Roe, unpublished results]. No data are available on the effect of the substitution by hydroxyl groups in the side-rings, and although the spectrographic evidence below suggests that in the phenol the hydroxyl groups are substituted in the anthracene nucleus, it does not eliminate the possibility of substitution in the side-rings.

In order to obtain further information as to the position of the hydroxyl groups in the phenol, the absorption spectrum of its quinone has been examined and compared with that of 1:2:5:6-dibenzanthraquinone (Fig 1). The main absorption band of the latter at 2962 Å. is shifted to 3042 Å. in the dihydroxy-derivative and its 2375 Å. band is similarly shifted to 2484 Å. There are greater differences in the long-wave region however, the band at 3900 Å. in 1:2.5·6-dibenzanthraquinone being replaced by one at 4535 Å in its dihydroxy-derivative.

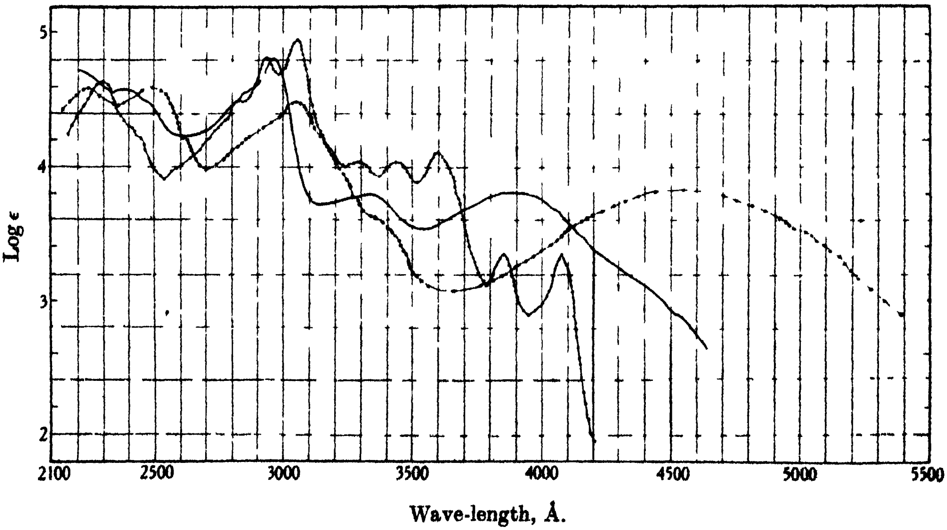


Fig. 1. •—• Dihydroxy-1:2:5:6-dibenzanthracene. o—o Dihydroxy-1:2:5:6-dibenzanthraquinone. —— 1:2:5:6-Dibenzanthraquinone.

These spectra may be compared with those of anthraquinone and its 2:6- and 1:5-dihydroxy-derivatives (Fig. 2). The spectrum of 2:6-dihydroxyanthraquinone is similar in form to that of anthraquinone and is shifted 200–300 Å. towards the visible. 1.5-Dihydroxyanthraquinone presents a different type of spectrum. The short-wave bands are shifted slightly towards the visible compared with anthraquinone, but the band at 3230 Å in the latter is replaced by a band at 4230 Å in the 1.5-dihydroxy-compound, a difference of 1000 Å.

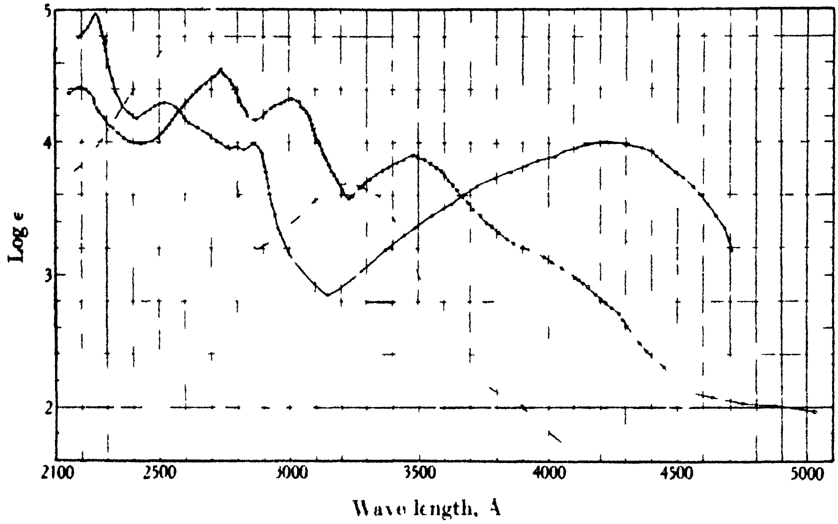


Fig. 2. —— Anthraquinone ● ● 1:5-Dihydroxyanthraquinone
○ ○ 2:6-Dihydroxyanthraquinone

Table 3. *Wave-lengths of maxima, with extinction coefficients*

λ = wave length in Å., ε = log $\frac{I_0}{I} / l\,c$

c = concentration in g. mol. per l., l = cm.

λ	log ε	λ	log ε
Dihydroxy-1:2:5:6-dibenzanthracene		9:10-Anthraquinone	
2298	4.64	2514	4.69
2823	4.49	2711	4.19
2928	4.81	3230	3.69
3045	4.95		
3289	4.04	2:6-Dihydroxy-9:10-anthraquinone	
3430	4.04		
3590	4.12	2190	4.41
3848	3.34	2733	4.54
4070	3.35	3004	4.33
		3475	3.90
Dihydroxy-1:2:5:6-dibenz-9:10-anthraquinone		1:5-Dihydroxy-9:10-anthraquinone	
2240	4.59	2253	4.97
2484	4.59	2515	4.30
3042	4.49	2768	4.00
4535	3.83	2860	3.99
1:2:5:6-Dibenz 9.10-anthraquinone		4230	4.00
2375	4·58		
2962	4.81		
3328	3.79		
3900	3.81		

This change is similar to that occurring in the spectrum of the dihydroxy-1:2:5:6-dibenzanthraquinone derived from the phenol when compared with its parent 1:2:5:6-dibenzanthraquinone and it suggests that the hydroxyl groups in the phenol may occupy the 1:5-positions of the anthracene nucleus, i.e. that the phenol isolated from rabbit urine is 4:8-dihydroxy-1:2:5:6-dibenzanthracene.

Tests for carcinogenic activity

The dihydroxy-1:2:5:6-dibenzanthracene was not sufficiently soluble in benzene to be applied by painting to mouse skin in that solvent. Experiments were made with an acetone solution of the substance (0·1 %), with which the backs of 10 mice were painted twice weekly. None of the mice developed tumours although one mouse survived the treatment for 16 months and another for 18½ months. In another experiment 10 mice were injected with 2 doses each of 1 mg. of the phenol suspended in olive oil, with an interval of one week between injections. All the mice died without tumours between the 51st and 119th day following the injection. This is in agreement with the findings of Dobriner *et al.* [1939].

DISCUSSION

It has unfortunately been necessary to interrupt this work before the suggested structure of the dihydroxydibenzanthracene could be tested by synthesis and also before making a complete examination of the excretion of dibenzanthracene in the rat.

Rats and rabbits produce different products from anthracene [Boyland & Levi, 1935; 1936] and also react differently to 1:2:5:6-dibenzanthracene. The injection of 1:2:5:6-dibenzanthracene into rats readily produces tumours at the site of injection while similar injections into rabbits do not [Burrows & Boyland, 1938]. It is possible that this difference in response is due to a difference in the metabolism of the carcinogen analogous to that found in anthracene metabolism.

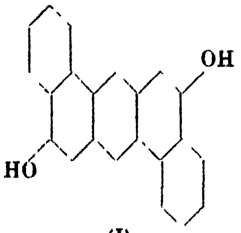
The non-carcinogenic phenol isolated from the urine of rabbits which were fed with the carcinogenic 1:2:5:6-dibenzanthracene may have been present as a tetrahydroxytetrahydro-1:2:5:6-dibenzanthracene or the corresponding glycuronide if the metabolism of dibenzanthracene is similar to that of anthracene. In that case the dibenzanthracene derivative must be less stable than the corresponding dihydroxydihydroanthracene which was converted into 1-hydroxyanthracene on gentle heating with dilute acid.

The positions of the hydroxyl groups in the molecule of the phenol cannot definitely be decided. The dihydroxydibenzanthracene even in alkaline solution was stable to air and did not give colour reactions with ferric chloride. It is therefore improbable that the hydroxyl groups are attached to adjacent carbon atoms or to carbon atoms in the *p*-positions in a benzene ring.

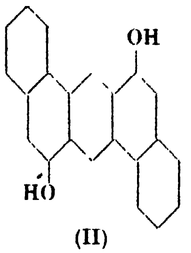
The fact that oxidation with $KMnO_4$ did not yield anthraquinone tetracarboxylic acid indicates that the hydroxyl groups are not in the side rings of the dibenzanthracene molecule. Cason & Fieser [1940] have synthesized 4′:8′-dihydroxy-1:2:5:6-dibenzanthracene which they describe as being probably identical with the substance excreted when dibenzanthracene is injected into rats and mice. This differs from the product excreted by rabbits which Cason & Fieser [1940] tentatively suggest is 3′:7′-dihydroxy-1:2:5:6-dibenzanthracene.

The formation of the diacetoxy-, dimethoxy- and dihydroxy-dibenzanthraquinone excludes the 9 and 10 positions. The non-formation of a boracetic ether suggests that neither of the hydroxyl groups is in the *peri* position to the quinone groups. The ease with which the dihydroxyquinone reacts with dimethyl sulphate is also in agreement with the hypothesis that the hydroxyl groups are in 3:7 positions. On the other hand the spectrographic evidence suggests that the compound might be 4:8-dihydroxy-1:2:5:6-dibenzanthracene.

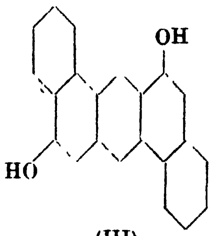
Possible formulae for the excretion product therefore include 3:7-dihydroxy-1:2:5:6-dibenzanthracene (I), 4:8-dihydroxy-1:2:5:6-dibenzanthracene (II) and 3:8-dihydroxy-1:2:5:6-dibenzanthracene (III).



(I)



(II)



(III)

SUMMARY

1. The administration of 1:2:5:6-dibenzanthracene or 3:4:5:6-dibenzcarbazole to rats or rabbits causes an increase in the excretion of ethereal sulphate in the urine.

2. A phenolic derivative, dihydroxy-1:2:5:6-dibenzanthracene was isolated from the urine of rabbits fed on a diet containing 1:2:5:6-dibenzanthracene. This phenol has been characterized and several derivatives prepared.

We are indebted to the British Empire Cancer Campaign for their support of this work, and to the Sir Halley Stewart Trust and the International Cancer Research Foundation for generous grants.

We wish to thank Messrs Hoffman La Roche for a gift of 3:4:5:6-dibenzcarbazole.

REFERENCES

Boyland & Levi (1935). *Biochem. J.* **29**, 2679.
—— —— (1936). *Biochem. J.* **30**, 728.
—— —— (1938). *Chem. Ind.* **16**, 446.
Burrows & Boyland (1938). *Amer. J. Cancer*, **32**, 367.
Cason & Fieser (1940). *J. Amer. chem. Soc.* **62**, 2681.
Claude (1937). *Amer. J. Cancer*, **31**, 100.
Cook (1931). *J. chem. Soc.* p. 2529.
Dobriner, Rhoads & Lavin (1939). *Proc. Soc. exp. Biol., N.Y.*, **41**, 67.
Fisher (1936). Statistical Methods for Research Workers, 6th ed. Edinburgh.
Folin (1905). *J. biol. Chem.* **1**, 131.
Mayneord & Roe (1935). *Proc. roy. Soc.* A, **152**, 299.
Polson (1936). *J. Path. Bact.* **42**, 537.

19. VITAMIN B_1 AND BACTERIAL OXIDATIONS

1. DEPENDENCE OF ACETIC ACID OXIDATION ON VITAMIN B_1

By J. H. QUASTEL and D. M. WEBLEY

From the Biochemical Laboratory, Cardiff City Mental Hospital

(*Received 29 November 1940*)

Vitamin B_1 is known to be a necessary factor for the growth of certain bacteria [Knight, 1937, 1, 2; cf. also Tatum *et al.* 1936; Barron & Lyman, 1939; Nilsson *et al.* 1938]. Such bacteria can be grown on media containing extremely minute quantities of the vitamin, and may even be trained to grow in the absence of the vitamin [Wood *et al.* 1938], in which case the organisms apparently may acquire the ability to synthesize it [Silverman & Werkman, 1939, 2]. The metabolism of bacteria grown on vitamin B_1-deficient media may be greatly enhanced by the addition of small quantities of the vitamin to suspensions of the organism. This has been shown by Hills [1938] in the case of *Staphylococcus aureus*, and by Silverman & Werkman [1938] in the case of propionic acid bacteria. The former author reported a marked stimulation of the oxidation of pyruvate and of the anaerobic breakdown of pyruvate by *Staphylococcus* on the addition of the vitamin. Silverman & Werkman [1938; 1939, 3] found that the rate of anaerobic breakdown of pyruvic acid by propionic acid bacteria and by *Lactobacillus mannitopoeus* is increased on the addition of the vitamin to washed suspensions of the organisms.

We have carried out a systematic study of the effects of vitamin B_1 on the oxidative powers of propionic acid bacteria grown on vitamin B_1-deficient media and of the various factors which influence the action of the vitamin. It is proposed to deal in this paper with the effects of the vitamin on various oxidations stimulated by these bacteria. We have already reported that the vitamin appears to be essential for the oxidation of acetic acid by these organisms [Quastel & Webley, 1939].

Preparation of vitamin B_1-deficient propionic acid bacteria

We have used a strain of propionic acid bacteria obtained from the National Collection of Type Cultures (Ref. No. 4759). The organism is grown on Petri dishes containing a medium deficient in vitamin B_1. This medium is prepared as follows. A mixture of 20 g. 'Difco' peptone, 6 g. NaCl and 40 g. agar-agar is dissolved in 1 l. of distilled water, the mixture being warmed until solution has taken place. *N* NaOH solution is now added until the *p*H is 9·0, using bromothymol blue as indicator, and the mixture is autoclaved at 125° for 1 hr. The medium is finally filtered through absorbent cotton wool and the *p*H adjusted to 6·6. This medium may be autoclaved many times without its consistency, after cooling in Petri dishes, being appreciably affected. Occasionally we have found it necessary to autoclave the medium a second time at *p*H 9·0 to reduce the vitamin B_1 content to the low levels required for the preparation of the vitamin-deficient bacteria.

The plates are spread with an 18 hr. culture of the propionic acid bacteria which have been previously grown on the vitamin-deficient medium. The organism is grown aerobically at 37° for 18 hr., after which it is washed off the plates with sterile 0·16 *M* saline. The suspension of organisms is washed twice with saline by centrifuging. It is finally suspended in saline and stored at 0°. 1 ml. of the suspension is usually made to contain about 10 mg. dry weight of the bacteria.

Measurement of the respiration of the bacteria

This is accomplished with the use of the Barcroft, and Warburg, respiratory apparatus. 1 ml. of the bacterial suspension is usually employed and to this are added solutions of phosphate buffer, substrate etc., to produce a final volume of 3 ml. The measurements of O_2 uptake are carried out at 37° at *p*H 7·4 in an atmosphere of air. The experiments are generally run for 1 hr.

Measurement of the weight of bacteria

This is carried out by means of the Spekker photoelectric absorptiometer. The opalescence of a known weight of the bacteria suspended in saline is measured on the instrument, using a standard light filter, and a calibration curve is drawn relating opalescence to bacterial weight. The curve is linear for densities of the organism not exceeding 0·25 mg. dry weight per ml. With the aid of this curve it is a simple matter at the termination of each experiment to dilute the contents of the respiration flask until a suitable density of the organism is obtained, to determine the opalescence on the absorptiometer and to estimate from the curve the weight of bacteria present in the suspension.

The effect of vitamin B_1 on the oxidation of acetate by vitamin B_1-deficient propionic acid bacteria

Acetic acid is known from the work of Stone *et al.* [1936] to be a hydrogen donator in presence of propionic acid bacteria. Little is known of the metabolism of acetic acid in presence of these bacteria, but Wood *et al.* [1937] conclude that their evidence, derived from analyses of the products of glucose fermentation, points to the conversion of acetic acid into succinic acid.

The addition of acetate to suspensions of vitamin B_1-deficient propionic acid bacteria increases the rate of O_2 uptake. The further addition of vitamin B_1 results in a large acceleration of the rate of oxidation of acetate. A typical result is shown in Table 1. The rate of O_2 uptake by the bacteria in the presence of acetate and vitamin B_1 is linear. As the oxidation proceeds, the *p*H of the solution in the respiration flask steadily increases, sodium bicarbonate and carbonate being formed. The vitamin has but little effect on the respiration of the bacteria in the absence of added substrates.

Table 1

Each manometer vessel contained 1·0 ml. suspension of freshly washed and freshly grown *B. acidi propioni*, 0·5 ml. *M*/5 sodium phosphate buffer *p*H 7·4, the amounts of substrates and vitamin B_1 stated below and 0·16 *M* saline to bring the volume to 3 ml. O_2 uptakes were determined in an atmosphere of air at 37° for a period of 2 hr.

Substrate added	Vitamin B_1 added μg.	O_2 uptake in 2 hr. μl.
Nil	Nil	197
0·3 ml. *M*/10 Na acetate	Nil	563
Nil	0·25	245
0·3 ml. *M*/10 Na acetate	0·25	1202

Variation of the velocity of O_2 uptake by propionic acid bacteria in presence of acetate with the concentration of vitamin B_1

The velocity of respiration of the vitamin B_1-deficient bacteria in presence of 0·01 *M* sodium acetate rises sharply as the concentration of vitamin B_1 present is increased. A maximum is reached at a concentration of approximately 30 μg. vitamin B_1 per 100 ml., i.e. 7×10^{-7} *M*. The velocity of O_2 uptake in presence of acetate and in absence of added vitamin B_1 is variable, depending on the conditions of culture and age of the organism. Usually the addition of acetate to a suspension of vitamin B_1-deficient bacteria increases the rate of O_2 uptake, the increase being most probably determined by the small quantities of the vitamin still present in the organism. The variation of the rate of O_2 uptake of the vitamin-deficient bacteria in presence of 0·01 *M* sodium acetate with variation of the amounts of vitamin B_1 added to the suspension of cells is shown in Fig. 1. It will be noted that a large increase in the rate of respiration in the presence of acetate is secured by the addition of only 0·1 μg. vitamin B_1 to the 3 ml. of cell suspension in the respiration flask.

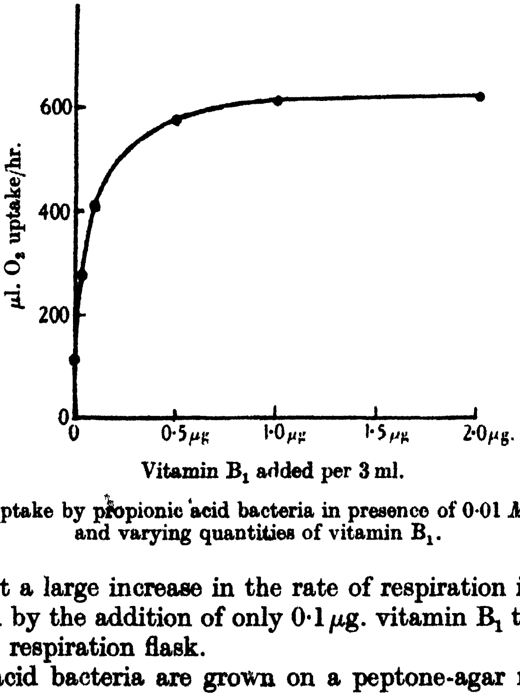


Fig. 1. O_2 uptake by propionic acid bacteria in presence of 0·01 *M* Na acetate and varying quantities of vitamin B_1.

If propionic acid bacteria are grown on a peptone-agar medium to which vitamin B_1 has been added, suspensions of these bacteria oxidize acetate at a much greater rate than holds when the bacteria are grown on vitamin B_1-free medium. Moreover, the addition of the vitamin to suspensions of bacteria grown on a vitamin B_1-rich medium does not increase the rate of oxidation of acetate.

O_2 uptake and bacterial count

The increased O_2 uptake secured by the addition of vitamin B_1 to suspensions of vitamin B_1-deficient bacteria in presence of acetate is not due to multiplication of the organisms in the respiration flask under our experimental conditions. This is shown by adding the same number of cells to a phosphate-acetate medium, in the absence and in the presence of vitamin B_1, measuring the O_2 uptakes at 37° for 1 hr. and making bacterial counts at the end of this period using a blood-counting chamber. A representative result is shown in Table 2.

Table 2

Conditions as in Table 1

Substance added to 1 ml. bacterial suspension	µl. O_2 consumed	No. of bacterial cells present at end of experiment
Nil	120·9	$10·6 \times 10^9$
0·25 µg. vitamin B_1	152·6	$9·4 \times 10^9$
0·01 *M* Na acetate	380·0	$9·8 \times 10^9$
0·01 *M* Na acetate + 0·25 µg. vitamin B_1	821·3	$9·5 \times 10^9$

Oxidation of acetic acid by propionic acid bacteria

Analyses were made of the acetic acid remaining in the respiration flask after the consumption of O_2 by the bacterial suspension, and the amount of acetic acid which had disappeared was compared with the amount of O_2 taken up.

Acetic acid was estimated in the following manner. A mixture of 3 g. anhydrous Na_2SO_4 and 1·5 ml. H_2SO_4 (2 parts conc. H_2SO_4 to 1 of water) was placed in a small pyrex flask and to this were added 2 ml. of the fluid in the respiration flask after the bacteria had been removed by centrifuging. The volume was made up to 6 ml. with distilled water and the flask was connected to a worm condenser and then to a Liebig condenser, ground glass joints being used throughout. The worm condenser and its outlet to the Liebig condenser were swathed with cotton wool. The end of the Liebig condenser dipped into 30 ml. of distilled water contained in a receiving flask. Distillation was carried out for 20 min. using a microburner under the distillation flask. The worm condenser prevented droplets of the fluid in the distillation flask from passing into the receiving vessel, but condensation of the steam containing the acetic acid took place only in the Liebig condenser. The microburner was removed after 20 min. distillation, the apparatus was disconnected, 5 ml. water were added to the distillation flask via the worm condenser and the apparatus was reconnected. Distillation was carried out for a further 20 min., after which the microburner was finally removed, and 30 ml. water were poured down the Liebig condenser (for washing purposes) into the receiving flask. The volume of fluid in this flask was finally 70 ml. The acetic acid present was titrated with CO_2-free N/100 NaOH, using a constant quantity of bromothymol blue as indicator. The method gave accurate and consistent results as long as the volumes mentioned above were adhered to.

Table 3

Substance added to the bacterial suspension	µl. O_2 consumed in 2 hr.	µl. O_2 consumed due to the acetate	µl. acetic acid utilized	Ratio $\frac{O_2 \text{ consumed}}{\text{Acetic acid utilized}}$
Exp. 1				
Nil	197	—	—	—
0·25 µg. vitamin B_1	245	—	—	—
0·01 *M* acetate	563	366	193	1·90
0·01 *M* acetate + 0·25 µg. vitamin B_1	1202	957	486	1·97
Exp. 2				
Nil	195	—	—	—
20 µg. cocarboxylase	221	—	—	—
0·01 *M* acetate	446	251	132	1·90
0·01 *M* acetate + 20 µg. cocarboxylase	1397	1176	591	1·99

Typical results giving the ratio of O_2 consumed by the bacteria to the acetic acid utilized are given in Table 3. The conditions were those quoted in Table 1. The acetic acid utilized by the bacteria is expressed in terms of gas (i.e. 1 g. mol. ≡ 22·4 l. gas).

The results show that the ratio of O_2 consumed to acetic acid utilized, whether vitamin B_1 has been added to the suspension of vitamin-deficient propionic acid bacteria or not, is approximately 2·0.

This value is to be expected if complete oxidation of acetic acid occurs according to the following equation:

$$CH_3COOH + 2O_2 = 2CO_2 + 2H_2O.$$

Effects of vitamin B_1 on the oxidation of fatty acids by vitamin B_1-deficient propionic acid bacteria

The lower fatty acids, formic, propionic and butyric, are oxidized by propionic acid bacteria, but the addition of vitamin B_1 has a much less marked effect on the oxidation of these acids than it has upon that of acetic acid. Representative results are shown in Table 4. It will be seen that whereas the rates of respiration in the presence of the fatty acids and in absence of added vitamin B_1 are approximately the same, the rates differ most markedly in the presence of the vitamin. The addition of the vitamin has but little effect on the oxidation of formate and butyrate, but is definite with propionate and most marked with acetate. The conditions of the experiments, typical results of which are shown in Table 4, are different from those mentioned earlier in that a mixture of magnesium ions and potassium ions was added to the solution in the respiration flask. The reasons for the addition of these ions will be given in a later paper.

Table 4

O_2 uptakes of vitamin B_1-deficient propionic acid bacteria in the presence of the Na salts of fatty acids and in the presence and absence of vitamin B_1. Each vessel contained 1 ml. freshly washed suspension of the bacteria, 0·5 ml. $M/5$ Na phosphate buffer pH 7·4, 0·1 ml. of a solution containing 0·01 M $MgCl_2$ and 0·024 M KCl, 0·3 ml. $M/10$ solution of the Na salt of the fatty acid, 0·2 ml. of the solution of vitamin B_1 and 0·9 ml. saline. Respiration measured in air at 37° Q_{O_2} = µl. O_2 uptake per hour per mg. dry wt. of bacteria.

	Q_{O_2}	
Substrate	In absence of added vitamin B_1	In presence of 4 µg. added vitamin B_1
Nil	10·5	12·4
0·01 M Na formate	22·5	24·9
0·01 M Na acetate	23·4	65·3
0·01 M Na propionate	20·7	37·8
0·001 M Na butyrate	19·0	25·1

It is of interest to note that Stone *et al.* [1936] have shown that resting propionic acid bacteria activate acetic and propionic acids to reduce methylene blue under anaerobic conditions, whilst relatively little or no activation takes place with formic and butyric acids.

Is acetate oxidized via pyruvate in presence of propionic acid bacteria?

It follows from the results quoted in Table 4 that not all oxidations brought about by vitamin B_1-deficient propionic acid bacteria are materially affected by the addition of vitamin B_1. This brings up the question as to whether the accelerating action of vitamin B_1 is primarily due to its effect upon the oxidation of an intermediate substance common to the breakdown products of acetic and propionic acids but not to those of formic and butyric acids. The obvious possible intermediate is pyruvic acid, the rate of oxidation of which by vitamin B_1-deficient propionic acid bacteria was found to be much increased by the addition of vitamin B_1, as is seen in Table 5.

Table 5

Conditions as in Table 4

Substrates added to bacterial suspension	Q_{O_2}
Nil	12·2
4 μg. vitamin B_1	14·5
0·01 *M* Na pyruvate	24·4
0·01 *M* Na pyruvate + 4 μg. vitamin B_1	42·8

The fact that propionic acid bacteria readily convert lactic acid into propionic acid makes it likely that the reverse process can take place, as pointed out by Stone *et al.* [1936]. It may be shown, with the use of vitamin B_1-deficient bacteria, that oxidation of propionic acid leads to the accumulation of a ketonic acid. When vitamin B_1 is added to the system the amount of ketonic acid accumulating is much diminished. Although the ketonic acid has not been isolated, the fact that it disappears in the presence of vitamin B_1 makes it most probable that the ketonic acid in question is pyruvic acid. This is shown much more clearly when glucose is used as the substrate for oxidation in presence of the vitamin-deficient bacteria. Here there is a rapid formation of a ketonic acid, the accumulation of which is greatly diminished by the presence of vitamin B_1. Its identity with pyruvic acid is made reasonably certain by the fact that Wood *et al.* [1937] have isolated this ketonic acid from the products of breakdown of glucose by the propionic acid bacteria. No other aldehydes or ketones were detected by these authors. The same phenomenon may be shown when lactate or glycerol is used as substrate for oxidation with the vitamin-deficient bacteria.

It is of some interest that the yield of pyruvic acid from lactic acid, when this is oxidized by the propionic acid bacteria, is much less than that from glucose. This shows that the oxidation of glucose does not depend on preliminary fission into lactic acid, and lends support to the view expressed by Wood *et al.* [1937] that the metabolism of glucose by the propionic acid bacteria follows the paths which are followed in muscle and yeast.

Representative results showing the rates of accumulation of pyruvic acid in the absence and presence of vitamin B_1 are given in Table 6. The yield of pyruvic

Table 6

Conditions as in Table 1

Substrates added to bacterial suspension	Vitamin B_1 added μg.	Dry wt. of bacteria mg.	Pyruvic acid liberated in 1 hr. μl.
Nil	Nil	10·5	Nil
Nil	4	10·5	Nil
0·01 *M* glucose	Nil	11·7	139·8
0·01 *M* glucose	4	11·7	86·0
0·01 *M* Na lactate	Nil	7·5	40·3
0·01 *M* Na lactate	4	7·5	6·7
0·01 *M* glycerol	Nil	10·3	26·9
0·01 *M* glycerol	4	10·3	10·0
0·01 *M* Na propionate	Nil	11·7	10·0
0·01 *M* Na propionate	4	11·7	Nil
0·01 *M* Na acetate	Nil	10·5	Nil
0·01 *M* Na acetate	4	10·5	Nil

acid is expressed in terms of μl. gas. It was estimated in the following manner. The fluid in the respiration flask at the end of the experiment was centrifuged. To 2 ml. of the supernatant fluid was added 1 ml. dinitrophenylhydrazine reagent (0·7 g. 2:4-dinitrophenylhydrazine hydrochloride dissolved in 250 ml. *N*·HCl). This mixture was allowed to stand for 10 min. at room temperature and then 2 ml. *N* NaOH were added to it. After allowing it to stand further for a few minutes with a little shaking, 5 ml. water were added. The solution was compared colorimetrically with solutions prepared in a similar way from known quantities of pyruvic acid (see Jowett & Quastel [1937]). The Spekker photoelectric absorptiometer was used for the colorimetric comparisons.

It will be observed from the results quoted in Table 6 that pyruvic acid accumulates during the oxidation of propionic acid by the vitamin-deficient bacteria in the absence of added vitamin B_1, as it does in the case of lactic acid and glucose. It is probable, therefore, that the stimulating action of vitamin B_1 on propionic acid oxidation, noted in Table 4, is partially if not wholly due to the effect of this vitamin on pyruvic acid oxidation. Yet in the case of the oxidation of acetic acid, in which the stimulating action of the vitamin is much more pronounced than in the case of propionic acid, there is no evidence at all of pyruvic acid accumulation in the absence of the vitamin (Table 6).

Experiments were carried out using semicarbazide as a fixative. Pyruvic acid accumulation may be shown to take place in the presence of semicarbazide when the sodium salts of lactic, succinic and propionic acids are oxidized by the vitamin B_1-deficient bacteria. None accumulates, however, when sodium acetate is oxidized under the same conditions. Typical results are shown in Table 7. The absence of any accumulation of a ketonic acid when the bacterial oxidation of acetic acid takes place in the presence of semicarbazide and in the absence of the vitamin throws doubt on the view that the stimulating action of the vitamin on acetic acid oxidation is connected with the oxidation of pyruvic acid as an intermediate. The following evidence may also be considered.

Table 7

Conditions as in Table 4; *M*/18 semicarbazide hydrochloride present; pH = 7·4; no vitamin B_1 added; 10·5 mg. dry wt. of bacteria.

Substrate present	Pyruvic acid liberated in 1 hr. μl.
0·01 *M* Na lactate	20·0
0·01 *M* Na succinate	20·2
0·01 *M* Na propionate	16·8
0·01 *M* Na acetate	Nil

(1) Examination of the results quoted in Table 3 shows that the ratio of O_2 consumption to acetic acid utilization is approximately 2, even in the absence of added vitamin B_1. If acetic acid were oxidized incompletely in the absence of added vitamin a ratio less than 2 would be expected. Thus, if it were oxidized to succinic acid the ratio would be 0·25, if to malic acid the ratio would be 0·5, and if to pyruvic acid the ratio would be 0·75. Since the experimental ratio is 2·0 the conclusion would be that acetic acid is completely oxidized in the absence of added vitamin B_1, but at a much smaller rate. Since traces of the vitamin must still remain in the bacteria the simplest conclusion accounting for the facts would be that vitamin B_1 is essential for the oxidation of the acetic acid molecule itself. It cannot be concluded from these experiments that pyruvic acid is not an intermediate in the oxidation of acetic acid, for it may be argued that in the

absence of added vitamin the oxidation of acetic acid is so slow that pyruvic acid is formed at a sufficiently feeble rate for it to be fully oxidized with the aid of the traces of vitamin B_1 still present in the bacteria. Admittedly, however, the absence of any accumulation of ketonic acid in the presence of a fixative such as semicarbazide throws doubt on the validity of this argument.

(2) The suggestion may be made that, in the absence of vitamin B_1, traces of intermediates accumulate during the oxidation of acetic acid by propionic acid bacteria and that these intermediates retard the oxidation of the fatty acid. Such intermediates may be succinic acid, *l*-malic acid and pyruvic acid. Experiment shows that the addition of small quantities of these substances to acetic acid has no retarding effect whatsoever on acetic acid oxidation. With succinic and *l*-malic acids an accelerating effect on the oxidation of acetic acid is observed. This phenomenon will be discussed in a later paper. With pyruvic acid no inhibition of acetic acid oxidation occurs, as shown by a typical result given in Table 8, in which pyruvic acid was still present at the end of the respiration period when the acetic acid analyses were made. There was thus ample ketonic acid present throughout the experiment to bring about the alleged retardation of acetic acid oxidation.

Table 8

Conditions as in Table 1

Substrates present	% acetate utilized in 1 hr.
0·01 *M* Na acetate	10·9
0·01 *M* Na acetate + 5 μg. cocarboxylase	41·5
0·01 *M* Na acetate + 0·0017 *M* pyruvate + 5 μg. cocarboxylase	46·0

(3) If acetic acid oxidation by propionic acid bacteria takes place via the intermediate production of pyruvic acid, it follows from the facts (*a*) that acetic acid is completely oxidized, (*b*) that no pyruvic acid accumulates, that the rate of removal of pyruvic acid by the bacteria should be greater than or at least equal to the rate of utilization of acetic acid under the same experimental conditions. Experiments designed to test this conclusion show that pyruvate is utilized by the bacteria at about the same rate as acetic acid. These results are shown in Table 9. Whilst this result is not inconsistent with the view that pyruvic acid may be formed during acetic acid oxidation, it follows (on this view) that pyruvic acid must be produced from acetic acid at an appreciable rate. It is, therefore, very surprising that none should appear in the absence of vitamin B_1.

Table 9

Conditions as in Table 4

Substrates added	Vitamin B_1 added μg.	Wt. of bacteria mg.	Substrate removed in 1 hr. μl. gas
0·01 *M* Na acetate	4	13·4	428
0·01 *M* Na pyruvate	4	13·4	504
0·0033 *M* Na acetate	4	10·5	178
0·0033 *M* Na pyruvate	4	10·5	181

(4) If a substrate is oxidized via pyruvic acid, it follows that during the oxidation of the substrate the enzyme dealing with pyruvic acid becomes either partially or wholly saturated with the ketonic acid. This will result in a lowering of the rate of utilization of any pyruvic acid initially added with the substrate, there being in effect less free enzyme available to cope with the added pyruvic

acid. Thus if a mixture of pyruvic acid and a substrate be presented to a bacterial suspension the rate of disappearance of the pyruvic acid will depend on the extent to which the pyruvic enzyme is saturated by the ketonic acid arising from oxidation of the substrate itself.

Experiments, of which typical results are given in Table 10, show that when pyruvic acid (0·0033 M) is mixed with lactic acid, succinic acid, fumaric acid or glycerol, vitamin B_1 being present throughout, there is almost complete inhibition of utilization by propionic acid bacteria of the pyruvic acid added initially. These highly inhibitory effects are quite consistent with the fact that these substrates give rise to small but demonstrable quantities of pyruvic acid during their oxidation (Tables 6, 7). They show that pyruvic acid is formed from them at a sufficient rate to saturate the enzyme involved. The admixture of propionic acid with pyruvic acid results in a definite inhibition of the rate of utilization of the latter, as would have been anticipated. The addition, however, of acetic acid to pyruvic acid results in no inhibition within the experimental error of these experiments. This result is opposed to the view that acetic acid is oxidized via pyruvic acid for the following reasons.

(*a*) Acetic acid, as already stated, is oxidized fast enough to produce pyruvic acid at a rate sufficiently high to saturate, partially or wholly, the pyruvic acid enzyme. Propionic acid, which is oxidized at a rate less than that of acetic acid, brings about a definite inhibition of the utilization of added pyruvic acid. The lack of inhibition due to acetic acid indicates that pyruvic acid is being produced from it either extremely feebly or not at all.

(*b*) If acetic acid is oxidized via pyruvic acid, succinic acid is presumably the first product of oxidation of the fatty acid. Succinic acid, however, inhibits the utilization of added pyruvic acid (Table 10) It follows, from the lack of inhibition due to acetic acid, that the rate of formation from it of succinic acid must be small. Considering the relatively rapid rate of oxidation of acetic acid, it seems unlikely, therefore, that it is oxidized to an appreciable extent via succinic and pyruvic acids.

Table 10

Conditions as in Table 1. 4 μg. vitamin B_1 present throughout.

Substrates added	$-Q_{\text{pyruvate}}$ (μl. pyruvate utilized per hr. per mg. dry weight bacteria)
0·0033 M Na pyruvate	14·5
+ 0·01 M Na *dl*-lactate	0·0
+ 0·01 M Na succinate	2·3
+ 0·0066 M Na succinate	3·9
+ 0·0033 M Na succinate	10·0
+ 0·01 M Na fumarate	0·0
+ 0·0066 M Na fumarate	0·0
+ 0·0033 M Na fumarate	1·4
+ 0·08 M Na malonate	15·1
+ 0·01 M Na formate	17·3
+ 0·01 M Na acetate	14·7
+ 0·01 M Na propionate	11·5
+ 0·01 M Na butyrate	15·4
+ 0·01 M Na α-glycerophosphate	14·1
+ 0·01 M glycerol	0·9

It will be noted from Table 10 that formic acid and butyric acid do not inhibit the utilization of pyruvic acid, nor is the latter affected by the addition of malonic acid or α-glycerophosphoric acid.

The conclusions to be drawn from these experiments are:

(1) Vitamin B_1 is necessary for the oxidation of acetic acid by propionic acid bacteria.

(2) There is as yet insufficient evidence to show that acetic acid is oxidized via pyruvic acid in presence of propionic acid bacteria, the available evidence being against this conclusion.

Oxidation of substrates other than fatty acids by propionic acid bacteria

As may be anticipated, those substrates the oxidations of which are known to proceed through pyruvic acid as an intermediate are oxidized by vitamin B_1-deficient propionic acid bacteria at rates[1] which are increased by the addition of vitamin B_1 to the bacterial suspensions. Typical results are shown in Table 11.

Table 11

Conditions as in Table 1

Substrates added	Q_{O_2}	
	In absence of added vitamin B_1	In presence of 4 μg. vitamin B_1
Nil	13·1	16·6
0·01 *M* Na pyruvate	20·5	38·8
0·01 *M* Na *dl*-lactate	35·6	52·8
0·01 *M* Na succinate	29·9	50·2
0·01 *M* Na fumarate	28·3	44·6
0·01 *M* Na *l*-malate	18·9	26·1
0·01 *M* glucose	23·5	34·3
0·01 *M* fructose	28·4	32·2
0·01 *M* Na *l*-glutamate	18·9	25·9
0·01 *M* Na α-ketoglutarate	16·5	18·1
0·01 *M* *dl*-alanine	16·6	20·6
0·01 *M* glycerol	28·8	35·7
0·01 *M* Na α-glycerophosphate	16·9	18·8
0·01 *M* Na hexosediphosphate	21·0	21·0
0·01 *M* Na citrate	15·8	16·8
0·01 *M* Na β-hydroxybutyrate	18·4	22·7

The oxidation of lactic acid by vitamin B_1-deficient propionic acid bacteria is stimulated by the addition of vitamin B_1 to an extent comparable with that occurring with the oxidation of pyruvic acid. Similar large accelerating effects due to the vitamin are to be noted with the oxidation of succinic and fumaric acids. It is, however, remarkable that the rate of oxidation of *l*-malic acid is less than that of fumaric acid and that the effect of vitamin B_1 addition is not nearly so pronounced as with succinic and fumaric acids. This result throws doubt on the generally accepted view that fumaric acid oxidation necessarily proceeds through the intermediate formation of malic acid. The fact that the rate of oxidation of fumaric acid may exceed that of malic acid has been noted before [Quastel & Wheatley, 1931] with *M. lysodeikticus* and *Ps. pyocyanea*.

This result, however, cannot be held as proof that fumaric acid oxidation, in the presence of propionic acid bacteria, proceeds independently of the formation of malic acid, as it is possible that fumarate may act catalytically in this oxidation. Thus, the following reactions may occur.

1. Fumaric acid $\rightleftarrows$ *l*-malic acid.
2. *l*-Malic acid + fumaric acid $\rightleftarrows$ oxaloacetic acid + succinic acid.
3. Succinic acid $\xrightarrow{O}$ fumaric acid.
4. Oxaloacetic acid $\rightarrow$ pyruvic acid + CO_2.

---

[1] I.e. the rates of oxygen uptake by the bacteria in presence of the substrates.

If reaction 2 is sufficiently rapid, a catalytic action of fumaric acid on malic acid oxidation will occur, its extent being dependent on the initial concentration of fumaric acid present.

A noteworthy fact is that α-ketoglutaric acid undergoes but little oxidation by propionic acid bacteria, its rate being scarcely influenced by the addition of vitamin B_1. Glutamic acid is oxidized at a somewhat faster rate and there is a definite, though feeble, stimulating action of vitamin B_1 on its oxidation. Alanine is but poorly oxidized, and vitamin B_1 has little effect on its rate of oxidation.

Both glucose and fructose are attacked by the vitamin B_1-deficient bacteria, but the addition of the vitamin has a much larger effect on the oxidation of glucose than upon that of fructose under the given experimental conditions.

It is interesting to note (Table 10) that whereas sodium α-glycerophosphate suffers little oxidative change by the bacteria, either in the presence or absence of vitamin B_1, glycerol is strongly attacked, the addition of vitamin B_1 having a marked effect on the rate of O_2 uptake. Sodium hexosediphosphate is oxidized at a rate which is appreciably faster than that of sodium α-glycerophosphate, but the rate is quite unaffected by the addition of vitamin B_1.

Sodium citrate and sodium β-hydroxybutyrate are oxidized but feebly, the addition of vitamin B_1 having little effect on the rates of O_2 uptake in presence of these substances.

Oxidation of ethyl and propyl alcohols by propionic acid bacteria

On testing methyl, ethyl and propyl alcohols as substrates for oxidation by vitamin B_1-deficient propionic acid bacteria, it was found that whereas methyl alcohol is not (or very feebly) attacked, ethyl and propyl alcohols are strongly oxidized. The addition of vitamin B_1 greatly increases the rate of oxidation of ethyl alcohol, but its effect on that of propyl alcohol is very much smaller. Typical results are shown in Table 12. The oxidation of ethyl alcohol leads to the appearance of a volatile acid, presumably acetic acid. Estimations show that the formation of this acid is greatly reduced when the oxidation of ethyl alcohol by the bacteria takes place in presence of vitamin B_1 (Table 13). Doubtless the stimulant action of vitamin B_1 on ethyl alcohol oxidation is partly due to its powerful effects on the oxidation of acetic acid formed as intermediary.

Table 12

Conditions as in Table 4

Substrate	Q_{O_2} In absence of added vitamin B_1	Q_{O_2} In presence of 4 μg. vitamin B_1
Nil	10·5	12·4
0·033 *M* methyl alcohol	11·5	13·9
0·033 *M* ethyl alcohol	32·2	44·1
0·033 *M* propyl alcohol	61·7	66·2

Table 13

Conditions as in Table 1

Substrate	μl. acetic acid in 1 hr. in absence of added vitamin B_1	μl. acetic acid in presence of 4 μg. vitamin B_1
0·03 *M* ethyl alcohol	201·6	47·0

Oxidation by propionic acid bacteria of substrates nearly related to acetic acid

It was of interest to examine the respiration of propionic acid bacteria in presence of substrates nearly related to acetic acid. Glycollic acid is oxidized at a low rate which is slightly increased by the addition of vitamin B_1. Glycine is oxidized at a higher rate with a small but definite accelerating action due to the addition of vitamin B_1. Neither of these substances is oxidized at a rate comparable with that of acetic acid in presence of the vitamin. Oxalic acid is oxidized either very feebly or not at all. These results are noted in Table 14.

Table 14

Conditions as in Table 4

Substrate added	Q_{O_2} In absence of added vitamin	Q_{O_2} In presence of 4 μg. vitamin B_1
Nil	10·5	12·4
0·01 *M* glycine	22·4	29·5
0·01 *M* Na glycollate	15·0	19·0
0·01 *M* Na oxalate	11·2	13·7
0·01 *M* Na acetate	23·4	65·3

Vitamin B_1 and the oxidation of α-ketobutyric acid

The addition of sodium α-ketobutyrate to vitamin B_1-deficient bacteria results in a rate of O_2 uptake approximately the same as that which occurs in the presence of sodium pyruvate. The addition of vitamin B_1, however, under the given experimental conditions results in little or no stimulation of the respiratory rate, in contrast to that which occurs with sodium pyruvate (see Table 15). Long & Peters [1939] found that with washed avitaminous pigeon brain the respiratory rate in presence of sodium α-ketobutyrate is stimulated to a small extent by the addition of vitamin B_1. Under different experimental conditions, which will be described in a later paper, we have found that there is a definite effect of vitamin B_1 on the oxidation of sodium α-ketobutyrate by the propionic acid bacteria. The effect, however, is much smaller than that occurring with pyruvic or acetic acid.

Table 15

Conditions as in Table 1

Substrate added	Q_{O_2} In absence of added vitamin B_1	Q_{O_2} In presence of 4 μg. vitamin B_1
Nil	13·1	16·6
0·01 *M* Na pyruvate	20·5	38·8
0·01 *M* Na α-ketobutyrate	19·0	21·7

Vitamin B_1 and cocarboxylase

Cocarboxylase is approximately as effective as vitamin B_1 in stimulating the rate of O_2 uptake by vitamin B_1-deficient bacteria in presence of sodium acetate. A characteristic feature of its activity, however, as is shown by typical results given in Table 16, is that its effects are a little less marked than those of vitamin B_1 at small concentrations and a little more marked at high concentrations. The fact

Table 16

Conditions as in Table 4. Substrate: 0·01 M Na acetate

	Q_{O_2}			
	0·3 μg.	1 μg.	5 μg.	10 μg.
Vitamin B_1	48·0	50·5	50·5	52·2
Cocarboxylase	37·6	41·8	53·4	56·2

that cocarboxylase activity exceeds that of vitamin B_1 at relatively high concentrations is evidence in favour of the conclusion that cocarboxylase, rather than vitamin B_1, is the actual catalytic agent. Silverman & Werkman [1939, 1, 4] have shown that propionic acid bacteria synthesize cocarboxylase from vitamin B_1. The fact that the activity of cocarboxylase in stimulating the respiration of propionic acid bacteria in presence of sodium acetate is feebler than that of vitamin B_1 at small concentrations may be attributed to a smaller rate of penetration of the pyrophosphoric acid ester into the bacterial cell.

Lipmann [1937] demonstrated the importance of cocarboxylase in the respiration of alkali-washed cells of *Bact. Delbruckii* in presence of sodium pyruvate. Barron & Lyman [1939] have found that cocarboxylase, rather than vitamin B_1, is the effective catalytic agent in the respiration of *Streptococcus haemolyticus* and of *M. gonorrhoeae* in a pyruvate medium.

Effects of components of vitamin B_1 on acetate oxidation by vitamin B_1-deficient propionic acid bacteria

It has been shown by Knight [1937, 1] that a mixture of 4-amino-5-aminomethyl-2-methylpyrimidine and 4-methyl-5-β-hydroxyethylthiazole is almost as effective as vitamin B_1 itself on the growth of staphylococci. Hills [1938] found that a mixture of the two components secures a large increase of O_2 taken up by vitamin B_1-deficient staphylococci in presence of sodium pyruvate. The components separately have no significant effect in raising the O_2 uptake.

A similar effect is found of the mixture of the two components on the oxidation of sodium acetate by vitamin B_1-deficient propionic acid bacteria. Typical results are shown in Table 17.

Table 17

Conditions as in Table 1. Substrate = 0·01 M Na acetate

Additions to the medium in the respiration flask	μl. O_2 uptake in 1 hr.
Exp. 1	
Nil	211·9
100 μg. pyrimidine component	215·6
100 μg. thiazole component	226·3
100 μg. pyrimidine component + 100 μg. thiazole component	435·0

Conditions as in Table 4. 0·01 M acetate throughout

Exp. 2	Q_{O_2}
Nil	28·8
0·2 μg. pyrimidine component	39·8
0·2 μg. thiazole component	27·5
0·2 μg. pyrimidine component + 0·2 γ thiazole component	60·5
0·2 μg. vitamin B_1	62·3

These results make it clear that propionic acid bacteria are able to synthesize vitamin B_1 from a mixture of the two components, even when these are present in low concentrations. Moreover, the results demonstrate that the components of vitamin B_1 are unable, separately, to exert catalytic effects on the oxidation of acetic acid by propionic acid bacteria. We have, however, found that under certain conditions the pyrimidine component can itself bring about an apparent stimulation of acetic acid oxidation (see, for example, exp. 2, Table 17). This finding is being further investigated. The phenomenon is probably similar to that described by Ochoa & Peters [1938] and explained by Westenbrink & v. Dorp [1940]. Silverman & Werkman [1939, 3] were unable to show a synthesis of vitamin B_1 from a mixture of the pyrimidine and thiazole components by propionic acid bacteria. Their pyrimidine component, however, differed from the one used in this work in having a $—CH_2Br$ group attached to the 5C atom of the pyrimidine ring instead of $—CH_2NH_2$. Conceivably, as Silverman & Werkman point out, linkage may not take place when the former group is attached to the pyrimidine ring.

SUMMARY

1. The presence of vitamin B_1 at low concentrations (e.g. *ca.* $10^{-7}M$) greatly increases the rate of oxidation of acetic acid by vitamin B_1-deficient propionic acid bacteria. The ratio of mol. oxygen consumed to mol. acetic acid utilized by these bacteria, in the presence and in the absence of vitamin B_1, is approximately 2·0, showing complete oxidation of acetic acid.

2. Formic, acetic, propionic and butyric acids are oxidized at about the same rate by vitamin B_1-deficient propionic acid bacteria. The addition of vitamin B_1 has little or no effect on the oxidation of formic and butyric acid, but has a definite stimulating action on that of propionic acid. This effect is much less than that observed with the oxidation of acetic acid.

3. Pyruvic acid accumulates during the oxidation of glucose, lactic acid, glycerol and propionic acid by vitamin B_1-deficient propionic acid bacteria. Its amount greatly diminishes in presence of the vitamin. No pyruvic acid accumulates when acetic acid is oxidized.

4. In presence of semicarbazide a ketonic acid (probably pyruvic acid) accumulates during the oxidation of lactic, succinic and propionic acids. It does not accumulate during the oxidation of acetic acid.

5. The rate of utilization of acetic acid by propionic acid bacteria in presence of vitamin B_1 is approximately equal to that of pyruvic acid.

6. The presence of lactic acid, succinic acid, fumaric acid or glycerol greatly inhibits the utilization of pyruvic acid, when this is added together with any of these substrates, by propionic acid bacteria in presence of vitamin B_1. Propionic acid also produces a definite inhibition of added pyruvic acid utilization, but acetic acid gives no inhibition within limits of experimental error.

7. The presence of vitamin B_1 greatly stimulates the rates of respiration by vitamin B_1-deficient propionic acid bacteria with the following substrates: pyruvic acid, lactic acid, succinic acid, fumaric acid, glucose, glycerol. Definite but much less powerful effects of the vitamin are shown in the oxidations of *l*-malic acid, *l*-glutamic acid, *dl*-alanine, glycollic acid, glycine and fructose. The following substances are oxidized either feebly or not at all by the bacteria, with little or no stimulating action of vitamin B_1: oxalic acid, citric acid, β-hydroxybutyric acid. Oxidation of α-glycerophosphoric acid and hexosediphosphoric acid takes place with little or no effect of added vitamin B_1 on their rates of oxidation. Oxidation of fumaric acid takes place at a greater rate than that of *l*-malic acid.

8. The stimulant action of vitamin B_1 may, in most cases, be attributed partially if not wholly to its effect on the oxidation of pyruvic acid formed as an

intermediate. The effect of the vitamin on acetic acid oxidation cannot at present be ascribed to this cause.

9. α-Ketobutyric acid is oxidized slowly by vitamin B_1-deficient propionic acid bacteria, there being little acceleration on addition of the vitamin.

10. Ethyl alcohol is oxidized by vitamin B_1-deficient propionic acid bacteria, the rate of oxidation in its presence being greatly stimulated by the addition of vitamin B_1. Acetic acid accumulates during the oxidation of the alcohol, its amount being greatly reduced in presence of vitamin B_1. Probably the accelerating action of the vitamin on ethyl alcohol oxidation is due to its powerful effects on the oxidation of acetic acid formed as intermediary. Propyl alcohol is vigorously oxidized by vitamin B_1-deficient bacteria, but there is relatively little accelerating effect due to the addition of vitamin B_1. Methyl alcohol suffers little oxidative change in the presence or absence of vitamin B_1.

11. The evidence is in favour of the conclusion that cocarboxylase is the effective catalytic agent.

12. The pyrimidine and thiazole components of vitamin B_1 are, separately, unable to catalyse the oxidation of acetic acid by propionic acid bacteria. Together they are as effective as vitamin B_1 or cocarboxylase. Thus linkage of the two components will take place in presence of propionic acid bacteria.

We are most grateful to the Medical Research Council for an assistance grant to one of us (D. M. W.) and for a grant in aid of the equipment of this laboratory.

We are also grateful to Dr Knight for gifts of the thiazole and pyrimidine components of vitamin B_1, to Dr H. Tauber and Messrs Merck for gifts of cocarboxylase, to Messrs Roche Products for a gift of vitamin B_1, and to Dr C. Long for a gift of the sodium salt of α ketobutyric acid.

REFERENCES

Barron & Lyman (1939). *J. biol. Chem.* **127**, 143.
Hills (1938). *Biochem. J.* **32**, 383.
Jowett & Quastel (1937). *Biochem. J.* **31**, 276.
Knight (1937, 1). *Biochem. J.* **31**, 966.
—— (1937, 2). *Biochem. J.* **31**, 731.
Lipmann (1937). *Nature, Lond.*, **140**, 25.
Long & Peters (1939). *Biochem. J* **33**, 759.
Nilsson, Bjalfre & Burström (1938). *Naturwissenchaften*, **26**, 284.
Ochoa & Peters (1938). *Biochem. J.* **32**, 1501.
Quastel & Webley (1939). *Nature, Lond.*, **144**, 633.
—— & Wheatley (1931). *Biochem. J.* **25**, 117.
Silverman & Werkman (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 823.
—— —— (1939, 1). *Proc. Soc. exp. Biol., N.Y.*, **40**, 369.
—— —— (1939, 2). *Iowa St. Coll. J. Sci.* **13**, 365.
—— —— (1939, 3). *Iowa St. Coll. J. Sci.* **12**, 107.
—— —— (1939, 4). *Enzymologia*, **5**, 385.
Stone, Wood & Werkman (1936). *Biochem. J.* **30**, 624.
Tatum, Wood & Peterson (1936). *Biochem. J.* **30**, 1898.
Westenbrink & v. Dorp (1940). *Nature, Lond.*, **145**, 464.
Wood, Anderson & Werkman (1938). *J. Bact.* **36**, 201.
—— Stone & Werkman (1937). *Biochem. J.* **31**, 349.

20. THE TRYPTOPHANASE-INDOLE REACTION

4. SOME OBSERVATIONS ON THE PRODUCTION OF TRYPTOPHANASE BY *ESCH. COLI*; IN PARTICULAR THE EFFECT OF THE PRESENCE OF GLUCOSE AND AMINO-ACIDS ON THE FORMATION OF TRYPTOPHANASE

By WILLIAM CHARLES EVANS, WILLIAM RICHARD CECIL HANDLEY[1] AND FRANK CHARLES HAPPOLD

From the Biochemical Laboratories, Department of Physiology, School of Medicine, Leeds, 2

(*Received 18 December 1940*)

Fildes [1938], referring to the claims of Happold & Hoyle [1936] that 'the failure to produce indole by cultures of *E. coli* grown in the presence of glucose is due to the non-production of a tryptophanase system', suggests that this is but a partial truth and that the tryptophanase system in the cells consists of two parts, a 'constitutive' part and an 'adaptive' part, of which only the 'adaptive' part is not produced in the presence of glucose. Fildes further states that suspensions of *E. coli* are always capable of catalysing the oxidation of tryptophan to indole even when grown in the absence of tryptophan or in the presence of glucose and tryptophan.

The main points of difference in technique and findings between the two series of investigations are: Fildes obtained his cells from cultures grown on a synthetic medium containing glucose and tryptophan and reported that indole was formed in these cultures and that the suspensions derived therefrom when incubated with tryptophan produced indole. Happold & Hoyle, having sought to explain the absence of indole formation which occurs in glucose bouillon cultures, grew their cells in this medium: indole was not produced in such cultures nor was there enzyme formation, since when non-viable preparations from these cells were placed in contact with tryptophan, indole was not formed, though similar preparations from bouillon cultures were active. The latter technique does not allow neo-enzyme formation to occur during the course of the experiment.

Both series of investigations have been repeated and are experimentally correct. Fildes' results may be due to neo-enzyme formation by the viable cell suspension in the presence of tryptophan as substrate and the absence of glucose. Certainly there are as yet no grounds for postulating a separate 'constitutive' tryptophanase system.

The experimental evidence which supports the above thesis was obtained as follows. Use was made of the previously observed phenomenon that the viability of washed cells of *E. coli* can be destroyed by the addition of a small amount of toluene immediately after the addition of the substrate and that this procedure inactivates only a proportion of the tryptophanase already formed, provided that the relative concentration of the toluene to the cells does not become too high. Treatment of the cells with toluene destroys their ability to cause carbohydrate breakdown.

---

[1] In part from the Department of Bacteriology, University of Birmingham.

EXPERIMENTAL

The Jebbs strain of *E. coli* (previously supplied by Prof. McIntosh) was used in all experiments. It was inoculated into media from weak suspensions prepared by suspending an agar colony of *E. coli* in 10 ml. sterile saline. The suspensions used for the enzyme work were gathered from 18–24 hr. cultures at 37°. Those taken from solid media were washed off with a mixture of 0·5 % saline and $M/60$ phosphate buffer (pH 7·0) and the cells separated from the suspending fluid by a high-speed centrifuge. The cells were washed twice in the saline phosphate mixture and finally suspended in it. Those taken from fluid media were separated from the medium, washed twice with the saline phosphate mixture and also suspended in the same manner. Glucose, where used, was added to the medium as a sterile solution to give final concentrations of 0·5–1·0 %. The details for growth experiments are given separately.

The non-production of the tryptophanase system in glucose bouillon agar cultures of E. coli

Washed suspensions from (*a*) two Roux bottles of bouillon agar and (*b*) two Roux bottles of bouillon agar + 1 % glucose were used. Suspensions and substrate were incubated separately at 37° for 15 min. before mixing. The following series of flasks was then set up, each flask containing 250 μg. tryptophan in 10 ml. of buffer mixture:

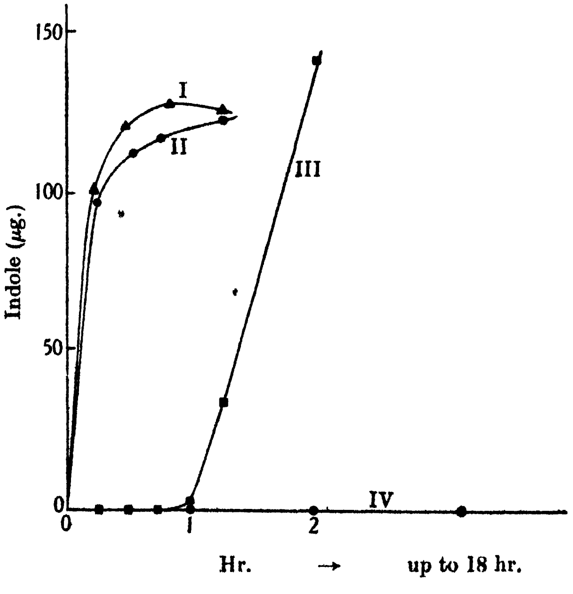


Fig. 1 Indole production from tryptophan by *E. coli*. I, *coli* cells from bouillon agar. II, *coli* cells from bouillon agar toluenated. III, *coli* cells from bouillon agar + 1% glucose. IV, *coli* cells from bouillon agar + 1% glucose toluenated.

Series 1. Substrate + 5 ml. of cell suspension from bouillon agar culture.
Series 2. Substrate + 5 ml. of cell suspension from bouillon agar culture + 0·2 ml. toluene.
Series 3. Substrate + 5 ml. of cell suspension from glucose bouillon agar culture.
Series 4. Substrate + 5 ml. of cell suspension from glucose bouillon agar culture + 0·2 ml. toluene.

The final turbidity of the suspensions was 2500 million cells per ml. The results are shown in Fig. 1.

The above series of results is characteristic of many similar experiments except that it represents one where there was no appreciable inactivation of the enzyme by the toluene. It will be observed that 87 % conversion of tryptophan into indole occurred as a result of the activity of cells grown in the absence of glucose, that in the glucose series there was practically no enzymic action after 1 hr. but that the reaction was 97 % complete after a further 2 hr. incubation, and finally that no indole was produced in the toluene-treated glucose agar suspensions. The only variations in results from many similar experiments of the above type are: (*a*) that inhibition of indole production in series (3) varies in lasting from 45 min. to 2 hr. after the addition of the substrate (this is probably due to the metabolism of stored carbohydrate); (*b*) that suspensions grown in the absence of glucose and treated with toluene are normally less active than their corresponding viable cells in series (1) by anything up to 60 %, depending on the cell concentration. This is shown in the following results, which are expressed in μg. units:

	30 min.	60 min.	120 min.	180 min.
(1) With 250 μg. tryptophan, after:	30 min.	60 min.	120 min.	180 min.
Washed suspension	20	30	50	60
Washed suspension + 0·2 ml. toluene	+	15	17	17
(2) With 1000 μg. tryptophan:				
Washed suspensions	Rapid rise to 119		—	100
Washed suspensions + 0·2 ml. toluene	Rapid rise to 55		—	62

The greatest variation has been obtained with washed cells grown in Fildes' medium + glucose and tryptophan. Frequently, we have obtained washed cells which have not produced more than 1–2 μg. indole from 250 μg. tryptophan in 16 hr.; frequently we have had rapid indole production with the washed cells, but always after a lag period, never have we obtained any indole formation from washed cells which have had toluene added after the addition of the substrate. An example of the second type of result is given in the following table:

Cells grown in 200 ml. Fildes' medium + glucose + tryptophan

	Indole in μg.			
	20 min.	30 min.	40 min.	60 min.
Washed cells + 250 μg. tryptophan	Nil	10	75	125
Washed cells + 250 μg. tryptophan + 0·2 ml. toluene	Nil	Nil	Nil	Nil

It will be observed that the results with the washed cells obviously fall on an autocatalytic curve as do those with the glucose bouillon agar suspensions and that adaptation to the substrate is taking place. The metabolism of stored carbohydrate is also progressing in the suspensions which are free from toluene. Cells grown in Fildes' medium alone (no tryptophan) are devoid of tryptophanase activity.

It has again been confirmed that fermenting glucose does not stop the tryptophan-tryptophanase indole reaction when the tryptophanase is already present before the glucose is added, and further that the tryptophan content of a medium does not decrease when *E. coli* is grown in it in the presence of glucose, at least whilst other amino-acids are present.

The former point is demonstrated by the following experiment. Twice-washed suspensions of organisms grown on bouillon agar were incubated in four 250 ml. flasks each containing 1 % glucose and 2 mg. of tryptophan per 50 ml. Two of the flasks contained 1 ml. of toluene in each flask. Glucose determinations showed that glucose was disappearing from the non-toluenated flasks during the course of the experiment. The agreement using this technique is not so good as when separate flasks are used for each determination, but the results are adequate for the purpose required:

Period of incubation	Indole produced per 10 ml. in μg.			
	Without toluene		With toluene	
	Flask (1)	Flask (2)	Flask (3)	Flask (4)
30 min.	51	41	21	22
1¼ hr.	83	69	37	44
3½ hr.	100	83	41	41
5¼ hr.	101	95	50	44

The non-disappearance of tryptophan from the culture medium when *E. coli* is grown in the presence of glucose, tryptophan and other amino-acids, i.e. when tryptophanase is not formed, was demonstrated by means of an eight-amino-acid medium [Evans *et al.* 1939] as follows:

Four flasks of the above eight-amino-acid culture medium were set up: (1) Basal medium uninoculated. (2) Basal medium inoculated with *E. coli*. (3) Basal medium + 0·5 % glucose uninoculated. (4) Basal medium + 0·5 % glucose inoculated with *E. coli*.

After 23 hr. incubation at 37°, 10-ml. samples from the flasks showed no change in reaction except that from flask (4) which was acid (*p*H 5). Tryptophan estimations on samples (by the method of Lugg [1937]) gave the following results:

	Tryptophan as g. per 100 ml.			
	Flask (1)	Flask (2)	Flask (3)	Flask (4)
After 23 hr.	0·0112	0·0019	0·0117	0·0111

Although the inoculated flasks (2) and (4) had both yielded a good growth of *E. coli* tryptophan had only disappeared from flask (2); this was the only flask which gave any reaction for indole and in this case it was a very strong reaction.

From the above experimental evidence it seems certain that the presence of tryptophan is essential for the production of tryptophanase, that where there is no indole formation in cultures containing glucose and tryptophan there is no tryptophanase production. Further, since resting bacteria do not apparently rest (but are capable of giving rise to neo-enzyme formation) the washed viable cell technique is unsafe as a method of analysis for the presence or absence of an enzyme at a given point in time.

Specific inhibition of indole production by E. coli *in the presence of phenylalanine or tyrosine and glucose in simple media*

There remains the fact, however, that under certain conditions, e.g. when grown in Fildes' medium + tryptophan, the inhibitory effect of glucose on the production of indole by cultures of *E. coli* is not complete, up to one-fifth of the indole produced in the absence of glucose being formed. This appears to be due to the peculiarities of the constitution of the medium.

It has been found that complete inhibition of indole formation in the presence of glucose and tryptophan in Fildes' medium can be obtained by adding 10 ml. of a 15 % gelatin solution per 50 ml. of Fildes' medium. Further, it was found that if the gelatin were replaced by a mixture of amino-acids representing those found in gelatin by analysis the same result was obtained. Each of the amino-acids found in gelatin hydrolysates was now added separately to Fildes' medium + glucose + tryptophan to a concentration of 0·01 %. Of the amino-acids used—

glycine, alanine, leucine, aspartic acid, glutamic acid, phenylalanine, cystine, lysine, arginine, histidine, proline and hydroxyproline—only phenylalanine and tyrosine (this was used later) completely inhibited indole production. At the concentration employed (0·01 %) the other amino-acids had no influence on the amount of indole formed. Further, it has been found that in the case of mono Na glutamate, leucine, valine, glycine, alanine, aspartic acid and histidine concentrations of 0·05, 0·10, 0·15 and 0·20 % have no effect on the production of indole in the presence of glucose. It has been demonstrated that phenylalanine and tyrosine do not affect the amount of indole produced from tryptophan in the absence of glucose. Numerous growth experiments have been carried out to determine the concentrations of phenylalanine and tyrosine necessary to complete the inhibition of indole production by glucose in Fildes' medium. The following relationships hold:

0·001 % tryptophan = 0·001–0·002 % tyrosine.
= 0·003–0·004 % phenylalanine.
0·002 % tryptophan – 0·003–0·004 % tyrosine.
– 0·005–0·006 % phenylalanine.
0·003 % tryptophan 0·004–0·005 % tyrosine.
– 0·007 % phenylalanine.

There is evidence here of a possible stoichiometric relationship between the tryptophan and the tyrosine and a less exact relationship between the phenylalanine and the tryptophan (the phenylalanine is a synthetic amino-acid).

The action of glucose + phenylalanine or tyrosine on preformed tryptophanase has also been studied.

Washed suspensions of *E. coli* grown on bouillon agar were prepared and added to flasks containing buffer solution *p*H 7·6 and 0·01 % phenylalanine or tyrosine sterilized by autoclaving as basal substrate. The following experimental flasks were set up: 30 ml. buffer solution and 30 ml. of cell suspension were placed in all the flasks:

(1) 1·5 ml. 1/1000 sterile tryptophan.
(2) 1·5 ml. 1/1000 sterile tryptophan + 0·05 % phenylalanine.
(3) 1·5 ml. 1/1000 sterile tryptophan + 0·05 % phenylalanine + 1 ml. toluene.
(4) 1·5 ml. 1/1000 sterile tryptophan + 0·05 % phenylalanine + 1 ml. 50 % sterile glucose.
(5) 1·5 ml. 1/1000 sterile tryptophan + 0·05 % phenylalanine + 1 ml. 50 % sterile glucose + 1 ml. toluene.

The tryptophan was added last to all the flasks, which were then incubated at 37°. 10-ml. samples were taken from each flask, ½, 1 and 3 hr. after the start of incubation. The results were as follows:

	Indole ½ hr.	Reaction		
		1 hr.	3 hr.	
Flask no.	µg.	µg.	µg.	Reaction
(1)	76	119	124	No change
(2)	76	120	125	,,
(3)	17	40	39	,,
(4)	76	110	108	Slightly acid
(5)	17	45	44	No change

Similar results obtained with tyrosine show that in the presence of phenylalanine and tyrosine preformed tryptophanase produced almost theoretical yields of indole from tryptophan and also that in the presence of fermenting glucose this yield is not reduced. It should be added that experiments performed for us by Mr H. Anderson show that cells of *E. coli* do not deaminate phenylalanine.

It is clear to us from these and other findings that the effect of the phenylalanine and tyrosine is not exerted through a competitive action upon the enzyme system when formed, but that it must be exerted during the stage of cell division when metabolism is in its most active phase. At the same time the specific effect of these two amino-acids, which show a structural relationship to tryptophan, raises points of interest in view of the findings of Baker & Happold [1940], that the integrity of the alanine group attached to the heterocyclic indole ring is essential to indole formation by suspensions of *E. coli*. It will also be noted that phenylalanine and tyrosine are two of the small group of amino-acids which are regarded as ketogenic in the mammalian metabolism of amino-acids.

It should be unnecessary to add that the term 'tryptophanase' is used, not to imply a single enzyme but to describe a coupled oxidative deamination and decarboxylation in an enzymic system which is specific for the tryptophan-indole reaction.

SUMMARY

It is further confirmed that the presence of tryptophan in a medium is essential for the production of the tryptophanase system by cultures of *E. coli*, and that the enzyme system does not exist as such in cells which have been grown in glucose bouillon agar or glucose tryptic digest agar, but that such cells when freed from glucose by washing will re-develop the enzyme system when left in contact with tryptophan.

Cells grown in complex amino-acid media plus glucose and tryptophan will produce some indole if phenylalanine and tyrosine are absent from such media. There is evidence of a stoichiometric relationship between the amount of tyrosine (?phenylalanine) which inhibits indole production and the tryptophan in media containing an increasing amount of tryptophan. This action is restricted in its effect to the growing cell. Phenylalanine and tyrosine alone or with glucose do not affect the tryptophanase activity of washed cells.

Acknowledgement is made to the Medical Research Council for a grant to one of us (F. C. H.).

REFERENCES

Baker & Happold (1940). *Biochem. J.* **34**, 657.
Evans, Happold & Handley (1939). *Brit. J. exp. Path.* **20**, 41.
Fildes (1938). *Biochem. J.* **32**, 1600.
Happold & Hoyle (1936). *Brit. J. exp. Path.* **17**, 136.
Lugg (1937). *Biochem. J.* **32**, 775.

21. HISTIDINE METABOLISM IN TOXAEMIA OF PREGNANCY. ISOLATION OF HISTAMINE FROM THE URINE OF PATIENTS WITH TOXAEMIA OF PREGNANCY[1]

By REGINE KAPELLER-ADLER

From the Institute of Animal Genetics and the Biochemical Laboratory of the Royal Infirmary, Edinburgh

(*Received 16 December 1940*)

In the course of various investigations it has been demonstrated that histidine is a normal constituent of the urine of pregnant women, that it appears in the urine at a very early stage of pregnancy and continues to be excreted during the whole of gestation [Kapeller-Adler, 1933; 1934; 1936]. The rate of histidine excretion seems to vary with the individual and to be dependent on the kind of food taken [Kapeller-Adler & Schiller, 1935]. No histidine is to be detected in the urine of pregnant animals [Kapeller-Adler & Herrmann, 1934]. Further studies revealed the existence of a relationship between the excretion of histidine and that of gonadotropic hormones in human pregnancy [Kapeller-Adler *et al.* 1935; 1936; 1937]. The increase in the gonadotropic hormones during human gestation was assumed to be the cause of the non-destruction of histidine so that this amino-acid passes the liver unaffected and, not being deaminated by the kidneys, is excreted in the urine. Whereas histidinuria is characteristic of normal human pregnancy a more or less distinct alteration in histidine metabolism occurs in toxic pregnancy. The clinical importance of this alteration is considered in a paper at present in the Press [Kapeller-Adler, 1940]. Briefly the results obtained in the study of 58 cases of toxaemia of pregnancy of various types indicate that histidinuria is not appreciably affected in cases of mild toxic pregnancy, but is considerably diminished in patients with serious symptoms of pre-eclamptic toxaemia; in these little or no histidine is to be found in the urine. So constant is this that a marked diminution in histidine excretion can be used as a diagnostic sign of severe toxaemia of pregnancy.

It seemed possible that in severe toxaemia the histidine instead of being excreted as such was being transformed into some other substance, probably one of pharmacological activity. In this connexion decarboxylation of histidine to histamine had obviously to be considered, and several attempts have been made to detect histamine in the urine from toxaemic patients as well as from cases of normal pregnancy. In this paper these attempts are described.

EXPERIMENTAL

The method used depended upon precipitation of the histamine with $Zn(OH)_2$ [compare Ackermann, 1939], decomposition of the precipitate with H_2S, extraction of the resulting alkaline solution with amyl alcohol, re-extraction of the histamine from the amyl alcohol with $N/10$ H_2SO_4 or $N/10$ HCl, evaporation of this solution *in vacuo* and preparation of the histamine picrate or flavianate.

---

[1] The substance of this paper was reported at a Meeting of the Biochemical Society in Edinburgh on 8 June 1940.

Exp. 1. 23 l. of urine of four patients with signs of severe toxaemia and no histidinuria were worked up. The urines were collected over chloroform and kept in the ice chest. Every 1000 ml. of the mixed urine were treated with 30 ml. of a saturated solution of freshly precipitated $Zn(OH)_2$ in ammonia (sp. gr. 0·880). After standing overnight the precipitate was filtered off, thoroughly washed with water and dried in air. The dry white powder was treated with dilute H_2SO_4 and freed from zinc by H_2S. The ZnS was filtered off and carefully washed with water. The filtrate was freed from H_2S by air-suction, made strongly alkaline with NaOH and the NH_3 was removed by distillation *in vacuo* at 40°. This solution, completely free from NH_3 and having *p*H 9·5, was concentrated *in vacuo* to about 100 ml. and shaken 6 times with pure amyl alcohol, 50 ml. being used for each extraction. The combined amyl alcohol extracts were shaken 5 times with $N/10$ H_2SO_4 (in later experiments HCl) using 20 ml. each time. To remove traces of amyl alcohol the sulphuric acid solution was vigorously shaken with 25 ml. of chloroform. The resulting liquid was concentrated to a small volume under reduced pressure and then tested for the presence of histamine as follows:

(*a*) With a small amount of this solution Pauly's diazo reaction [1904] was carried out and a strong positive result was obtained. This very sensitive test, although not specific for histamine, being given also by other iminazole derivatives, seemed however in this case to indicate the presence of histamine because other iminazole derivatives are insoluble in amyl alcohol.

(*b*) Another part of the solution obtained was subjected to the reaction of Zimmermann [1929]. This test, which is specific for histamine, also gave a strong positive result.

(*c*) To the main part of the liquid above described an excess of a concentrated solution of flavianic acid was added. After some time a pale yellow precipitate was formed. The M.P. was 256°. This precipitate was purified by repeated crystallizations from hot water. Ultimately, a fraction of a milligram of a yellow crystalline substance, insoluble in water, was obtained and this had M.P. 260°, not depressed on admixture with an authentic specimen of histamine diflavianate. Under the microscope the material appeared as bright yellow characteristic prisms showing great similarity with crystals of histamine diflavianate. On account of the strong positive result of the Zimmermann reaction in the fluid investigated and of the fact that the isolated flavianate showed many physical properties characteristic of histamine diflavianate it was assumed that the base present might indeed be histamine. It was of course necessary to obtain further evidence for that assumption.

Exp. 2. 34 l. of urine of several patients with signs of severe toxaemia of pregnancy and no histidinuria were worked up in the manner above described. The flavianate isolated had after repeated recrystallization M.P. 260°, not depressed on admixture with histamine diflavianate. Unfortunately, the yield of the material obtained was again very small, amounting only to a fraction of a milligram. Since the identification of the compound, so far carried out, was not at all satisfactory a further attempt was undertaken to procure a greater yield.

Exp. 3. This time it was decided to start from the urine of only two patients with signs of very severe toxaemia of pregnancy and no histidinuria at all in the hope that under these conditions histaminuria might be so considerable as to allow of histamine being isolated in larger amounts. 52 l. of urine from the two patients were treated in the way described, the only difference being that the amyl alcohol extracts were shaken with $N/10$ HCl instead of $N/10$ H_2SO_4. The liquid thus obtained was evaporated to dryness *in vacuo* after the traces of amyl alcohol had been removed by chloroform. A hydrochloride was obtained which showed strong positive Pauly and Zimmermann reactions. After recrystallization from hot alcohol prisms were obtained which, under the microscope, seemed very similar to crystals of histamine dihydrochloride. This hydrochloride was converted into a picrate which after several recrystallizations from hot water melted at 238–242° with decomposition and brown-black colouring. It showed no depression of M.P. when mixed with histamine dipicrate. This experiment yielded 2·7 mg. of the picrate which, like histamine dipicrate, was soluble in hot water and insoluble in ether. Under the microscope the crystals of the isolated picrate, consisting of yellow plates, closely resembled those of histamine dipicrate. It may be pointed out that histamine picrate as well as histamine flavianate give directly a strong positive diazo reaction without having been previously freed from picric or flavianic acid. Unfortunately, the amount of the picrate obtained was not large enough for a micro-analysis but X-ray powder photographs of it were kindly taken by Dr C. A. Beevers, Chemistry Department, Kings Buildings, Edinburgh.

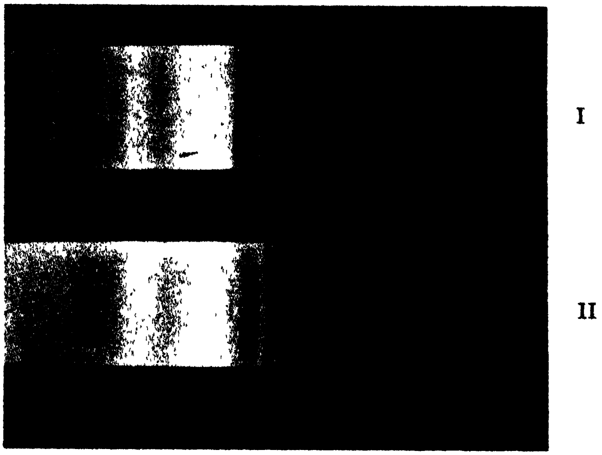


Fig. 1. Photographs of substance I (histamine dipicrate) and substance II (picrate isolated from toxic pregnancy urine).

Dr Beevers makes the following observation on these photographs:

(1) General features identical but II has some extra lines (continuous parts due to glass).

(2) I and II have at least nearly the same crystal structure. What differences there are may be due to (*a*) impurities in II crystallizing in quite a different way; (*b*) imperfect crystallization of I resulting in certain lines becoming blurred. This is supported by the fact that photograph I is generally more blurred than II.

(3) Lines are very numerous, showing a large unit cell (probably large molecules).

(4) Photographs were taken with Cu K X-rays (Ni filter to remove K_β), 2¾ hr. exposure, 25 mA., 60 kW.).

Exp. 4. Whereas in the previous experiments urines of women suffering from pre-eclamptic toxaemia were used for the isolation of histamine this time the examination of the urine of a woman with hyperemesis gravidarum for the presence of histamine was undertaken.

The urinary output of 6 successive days (3000 ml.) of a woman in the 10th–12th weeks of pregnancy with distinct signs of hyperemesis gravidarum and with almost no histidinuria was the starting material. The urine was daily precipitated with $Zn(OH)_2$ and the precipitate worked up in the manner described above, with only one slight modification. After the decomposition of the zinc salt by H_2S a few ml. of ammonia solution were added to the liquid containing dilute H_2SO_4 in order to ensure complete precipitation of ZnS. The precipitate was allowed to settle overnight and was then worked up according to exp. 3. The hydrochloride obtained showed strong positive diazo and Zimmermann reactions. In presence of a little dilute H_2SO_4 it was converted into the flavianate by means of a concentrated solution of flavianic acid. The bright yellow flavianate showed a strong positive diazo reaction and had M.P. 258°. On several recrystallizations a pure substance was obtained with constant M.P. 260°, not depressed on admixture with an authentic specimen of histamine diflavianate. The yield of the purified material was 10·9 mg. (Found (Weiler): C, 39·70; H, 3·6; N, 13·4%; required for $C_5H_9N_3(C_{10}H_6N_2SO_8)_2$: C, 40·58; H, 2·86; N, 13·26%.)

Exp. 5. 39 l. of urine from four normal pregnant women with histidinuria were worked up as in exp. 4. The hydrochloric acid solution finally obtained, although showing a positive diazo reaction, did not yield any histamine diflavianate with flavianic acid. Obviously only traces of histamine were present.

It may be mentioned that Ackermann [1939] has isolated 0·9 mg. histamine dipicrate from 1000 l. of normal urine. Because of the very small amount of the compound he could not identify it accurately.

DISCUSSION

The experiments recorded above indicate that whereas normal pregnancy is characterized by histidinuria, in severe toxaemia of pregnancy the excretion of histamine in the urine seems to replace that of histidine. It should be stressed that the amount of histamine isolated certainly represents only a small part of the amount which may really be present in toxaemic pregnancy urine, since losses in the preparation cannot be avoided and, apart from that, not all the histamine can be recovered.

There is no doubt that the histamine appearing in toxaemia of pregnancy can only be derived from histidine which is normally present in large amounts throughout gestation. Histamine is presumably formed from histidine by decarboxylation. Werle [1936; 1937; 1938] has found that animal tissues, especially the kidneys, contain an enzyme which can alter histidine to histamine by decarboxylation. This enzyme which he called histidine decarboxylase appears to be more effective in absence than in presence of oxygen. But there is still another enzyme in the kidneys with an antagonistic action tending to the destruction of histamine. This enzyme, histaminase, first described and isolated by Best [1929; 1930; 1932; 1935] is effective only in the presence of oxygen.

Although no evidence has been advanced as yet as to the occurrence of histidine decarboxylase in the human body, the above-reported isolation of histamine from toxaemic pregnancy urine seems to suggest the presence of such an enzyme in man. For the conversion of histidine which is excreted in rather large amounts throughout normal pregnancy into histamine appearing in the urine of women with different manifestations of severe toxaemia of pregnancy can only be explained if the presence of a histidine decarboxylase in the human body is assumed.

Accordingly two factors seem to be responsible for the velocity of formation of histamine in histidine metabolism, namely, the activity of the histidine decarboxylase and that of histaminase. In a recent paper Werle [1940] reports that the activities of both enzymes are not only dependent on the presence or absence of oxygen but far more on the presence of various inactivators which regulate their action.

Thus the occurrence of histamine in the metabolism of pregnant women will finally be determined by the balance between histidine decarboxylase and histaminase.

The significance of the isolation of histamine from toxaemic pregnancy urine for the explanation of the pathogenesis and of the course of toxaemia of pregnancy will be discussed in another place.

SUMMARY

1. A chemical method has been described for isolation of histamine from the urine.

2. Four attempts have been made to isolate histamine from the urine of women with various manifestations of toxaemia of pregnancy. Whereas in the first two experiments only fractions of a milligram of histamine diflavianate have been isolated, exp. 3 yielded 2·7 mg. of histamine dipicrate from 32 l. of urine of two women with very severe toxaemia, identified by melting point, mixed melting point, the Zimmermann reaction and an X-ray photograph; in exp. 4, 10·9 mg. histamine diflavianate have been isolated from 3000 ml. of urine of a woman with severe hyperemesis gravidarum and identified by a micro-analysis.

3. From 39 l. of normal pregnancy urine with normal histidine excretion the same method yielded no detectable quantity of histamine.

4. The origin of histamine and the factors responsible for its occurrence in toxic pregnancy are discussed.

I wish to express my gratitude to Prof. F. A. E. Crew and to Dr C. P. Stewart for kind hospitality. To Prof. R. W. Johnstone I am deeply indebted for permission to use the clinical material from his Ward in the Simpson Memorial Maternity Hospital, Edinburgh. My thanks are also due to Prof. G. F. Marrian for his helpful criticism and advice.

REFERENCES

Ackermann (1939). *Hoppe-Seyl. Z.* **259**, 32.
Best (1929). *J. Physiol.* **67**, 256.
—— (1930). *J. Physiol.* **70**, 349.
—— (1932). *Biochem. J.* **26**, 1365.
—— (1935). *Biochem. J.* **29**, 315.
Kapeller-Adler (1933). *Biochem. Z.* **264**, 131.
—— (1934). *Klin. Wschr.* **13**, 21.
—— (1936). *Klin. Wschr.* **15**, 1728.
—— (1940). *J. Obstet. Gynaec.* (in the Press).
—— & Boxer (1937). *Biochem. Z.* **293**, 207.
—— & Haas (1935). *Biochem. Z.* **280**, 232.
—— & Herrmann (1934). *Klin. Wschr.* **13**, 1220.
—— —— (1936). *Klin. Wschr.* **15**, 977.
—— & Schiller (1935). *Klin. Wschr.* **14**, 1790.

Pauly (1904). *Hoppe-Seyl. Z.* **42**, 510.
Werle (1936). *Biochem. Z.* **288**, 292.
—— (1937). *Biochem. Z.* **291**, 105.
—— (1938). *Biochem. Z.* **296**, 315.
—— (1940). *Biochem. Z.* **304**, 201.
Zimmermann (1929). *Hoppe-Seyl. Z.* **186**, 260.

Note added 20 February 1941. Further identification of histamine isolated from the urine of patients with toxaemia of pregnancy.

Since the above paper was submitted more histamine has been isolated from the urine of two toxaemic patients. 7700 ml. of urine of a patient with hyperemesis gravidarum yielded 34·6 mg. histamine diflavianate and 62·5 mg. of the same salt were prepared from 8500 ml. of urine of a woman suffering from serious pre-eclamptic toxaemia. In both cases the histamine diflavianate first obtained was purified and was then freed from flavianic acid by dilute hydrochloric acid and butyl alcohol, thus giving a pure histamine hydrochloride. This salt was converted again into the diflavianate which had M.P. 262–263°, not depressed on admixture with an authentic specimen of histamine diflavianate. (Found (Weiler): C, 39·71; H, 3·22; N, 12·96 %; required for $C_5H_9N_3(C_{10}H_6N_2SO_8)_2$: C, 40·58; H, 2·86; N, 13.26 %.)

Prof. A. J. Clark, Department of Pharmacology of the University, Edinburgh, kindly carried out biological tests with the preparations from both of these patients. In the case of the patient with hyperemesis the pure hydrochloride was used, whilst in the second case the hydrochloride contained traces of the flavianate. In tests on the guinea-pig's ileum, the guinea-pig's uterus and the rat's uterus the preparations from urine were identical in pharmacological action with similar amounts of authentic histamine.

ERRATA

Vol. 34, Nos. 10 and 11, page 1467

line 1 *after the words* ‘in suspension’

add ‘Sodium chloride is then added to the clear solution to give molar concentration’

Vol. 34, Nos. 10 and 11, p. 1470

			Probable limits of error	
centre of page	*for*	Ratio	$P=0{\cdot}95$	$P=0{\cdot}99$
		0·894	79–127 %	83·5–120 %
			Probable limits of error	
	read	Ratio	$P=0{\cdot}95$	$P=0{\cdot}99$
		0·894	83·5–120 %	79–127 %

22. EFFECT OF DIPHTHERIA TOXIN UPON TISSUE ENZYMES *IN VITRO*

By BENJAMIN ALFRED PETERS AND
ROBERT NEIL CUNNINGHAM

From Ham Green Hospital, Bristol

(*Received 25 October 1940*)

THE normal course of diphtheria is generally characterized by pronounced ketonuria and other symptoms indicating serious metabolic disturbances. Many of the regular pathological symptoms, e.g. circulatory failure, fatty changes in the heart, interference with its action and haemorrhage into the adrenals, are also capable of being traced back to a biochemical origin, whilst the last-named occurrence may obviously lead automatically to further chemical interference.

We have already shown [Peters & Cunningham, 1937] that the polyneuritic condition that occurs in the later stages is not due to induced vitamin B_1 deficiency, since the chemical changes associated therewith in the blood are not shown. Since there are no indications of lack of any other vitamins or dietary factors we conclude that the chemical disturbances are of a more profound origin. We have therefore turned our attention to a number of isolated tissue enzyme systems, and studied their behaviour in presence of diphtheria toxin.

The scope of our work embraces respiration, glycolysis, oxidations (including autoxidations) and phosphorus exchange.

Technique. For our work on respiration and oxidation we generally used the Warburg manometric technique, but macro- or semi-micro-analytical methods were substituted for the study of glycolysis and P interchange.

The toxin usually employed was a dried preparation containing 250,000 m.l.d. per g. (the gift of the Evans Biological Institute, to whom our thanks are due), free of preservatives, which had been found undesirable; it was used in large excess of the m.l.d., usually 1 or 2 mg.

Enzyme preparations were obtained as far as possible from heart muscle, which is known to be affected in diphtheria.

EXPERIMENTAL

Glycolysis

Finely chopped fowl's heart muscle was suspended in Ringer solution at pH 7·4 in a Krebs flask [Krebs & Henseleit, 1932], and incubated for 3–4 hr. at 38° in a stream of O_2 containing 5 % CO_2. Flasks were set up in pairs, one containing toxin. Lactic acid was determined before and after incubation by the method of Friedemann *et al.* [1927].

Table 1 shows typical results from a large number of experiments. The increases in lactic acid per dg. of dried tissue are practically identical with and without toxin. Pyruvate was never detected in the mixture after incubation. Anaerobic experiments in an atmosphere of N_2 with 5 % CO_2 also gave negative results.

Table 1. *Glycolysis*

2 g. tissue in 40 ml. Ringer medium (Ringer phosphate for Exp. 12). Glucose, 0·2%. Toxin 0·5 ml. (=25 m.l.d.) Burroughs Wellcome conc. solution, dil. 1 in 50.

Exp.	Incubation hr.		Tissue (g.) dry wt.	Lactic acid (mg.) Initial	Lactic acid (mg.) Final	Increase (mg./dg. dry tissue)
10	3	Control	0·3	1·7	2·4	0·29
		Toxin	0·3	2·0	2·1	0·03
12	3·5	Control	0·44	2·5	4·9	0·55
		Toxin	0·42	1·5	3·9	0·58

Tissue respiration

Slices of fowl's heart, suspended in phosphate Ringer at *p*H 7·4, were employed for a long series of manometric experiments in presence of glucose, glucose and citrate, glucose and lactoflavin, lactate, lactate and fumarate, and succinate. The manometer flasks were filled with pure O_2 and shaken for an hour at 38°. Parallel 'blank' experiments were run with the tissue but no substrate. The results indicated that toxin caused no regular alteration in Q_{O_2}.

Oxidizing systems

A number of the oxidase systems that have been shown to exist in the body were set up *in vitro* and the effect of diphtheria toxin examined on each of them under various conditions depending on the particular enzyme being studied; these included succinic, lactic, malic, β-hydroxybutyric, α-glycerophosphoric and pyruvic dehydrogenases, and amine, amino-acid and adrenaline oxidases. The autoxidation of adrenaline in buffer was also studied.

Cozymase, when required, was prepared from yeast by a method described by Green (private communication), and further purified with CuCl [Euler *et al.* 1936]. We are indebted to Messrs Roche Products Ltd. for the gift of a sample of cocarboxylase for use in the pyruvic dehydrogenase experiments.

Acetone-dried preparations supplemented by diaphorase were used in a few experiments with lactate, malate and pyruvate.

Oxidations were generally carried out in phosphate buffer at *p*H 7·2–7·4 (except where otherwise stated) and an atmosphere of pure O_2. Toxin was added direct to the enzyme in some experiments, but in others it was mixed with the enzyme (and often cozymase as well) on the previous day and the mixture left overnight in the ice-box to attempt to allow for the 'latent period' observed with the toxin *in vivo.*

Tables 2–9 show typical results for all these series of experiments. The total number of experiments is indicated at the head of each table.

Lecithin. The effect of the toxin was studied on the oxidation of lecithin in presence of glutathione [Hopkins, 1925]. Experiments were carried out in acetate buffer at *p*H 4·6, which was found to be the optimum reaction. Preliminary contact with the toxin for varying periods was tried with each reactant. Other proteins, named in Table 10, were used for comparative experiments to ascertain if they produced any effects similar to that of toxin.

The final volume of liquid in the manometer flasks was always 2 ml., about 1 ml. of enzyme suspension being used and the other reagents added in the concentrations or dry weights given. If cyanide was present the precautions of Krebs [1935] were followed with respect to the CO_2-absorbing liquid in the inner cups.

Table 2. *Succinic dehydrogenase*

1 ml. enzyme [Stotz & Hastings, 1937] from heart of pig or sheep; $0{\cdot}05\,M$ phosphate, *p*H 7·4; $0{\cdot}05\,M$ succinate; 1 mg. toxin overnight with enzyme. Twenty-three experiments.

Exp.		O_2 uptake in 30 min. (μl.) Control	Toxin
15	No methylene blue	276	290
	Methylene blue, 0·008 %	278	261
23	No selenite	339	342
	Selenite, $0{\cdot}5 \times 10^{-3}\,M$	19	17
16	HCN, $2{\cdot}5 \times 10^{-3}\,M$, methylene blue 0·008 %	50	45

Table 3. *Lactic, malic and β-hydroxybutyric dehydrogenases*

1 ml. lactic [Green & Brosteaux, 1936], malic [Green, 1936, 2], or β-hydroxybutyric dehydrogenase [Green *et al.* 1937], from pig or sheep heart; 0·025 % methylene blue, $0{\cdot}015\,M$ pyrophosphate; HCN, hydrazine or semicarbazide ($0{\cdot}015\,M$) as ketone fixative; toxin overnight with enzyme. Ninety-eight experiments.

Exp.	Substrate	O_2 uptake per hr. (μl.) Control	Toxin
46	Lactate	249	230
82*	Lactate	29	27
29	Malate	197	203
84*	Malate	24	24
79	β-Hydroxybutyrate	40	41

*Dried muscle enzyme with heart flavoprotein [Straub, 1939] as diaphorase.

O_2 uptake was not altered significantly by overnight contact between the toxin and the enzyme or enzyme-cozymase, presence or absence of pyrophosphate, or any particular ketone fixative.

Table 4. *α-Glycerophosphate dehydrogenase*

1 ml. enzyme [Green, 1936, 1] from guinea-pig skeletal muscle; $0{\cdot}04\,M$ α-glycerophosphate 2 mg toxin; *p*H 7·2. Two experiments.

Exp.		O_2 uptake per hr. (μl.) Control	Toxin
1	No methylene blue	43	42
2	Methylene blue, 0·075 %	170	168

Table 5. *Pyruvic dehydrogenase*

0·8 ml. enzyme [Lipmann, 1939] from skeletal or heart muscle; $0{\cdot}2\,M$ NaF, $0{\cdot}04\,M$ phosphate *p*H 7·2, $0{\cdot}04\,M$ pyruvate, 4 mg. adenylic acid (yeast), 3 mg. toxin; 1 mg. cozymase and 1 mg. cocarboxylase in side-bulb with pyruvate. Seven experiments.

Exp.		O_2 uptake per hr. (μl.) Control	Toxin
2		171	176
6	0·25 mg. lactoflavin (Roche) added	27	29

Table 6. *Amine oxidase*

1 ml. enzyme [Richter, 1937] from liver; 0·5 mg. toxin added in 0·9 % NaCl, $0{\cdot}001\,M$ HCN as ketone fixative. Seven experiments.

Exp.	Substrate	O_2 uptake per hr. (μl.) Control	Toxin
4	Tyramine, $0{\cdot}04\,M$	66	71
5	Adrenaline, $0{\cdot}005\,M$	84	87

Table 7. *Amino-acid oxidase*

1 ml. enzyme [Krebs, 1933] from guinea-pig kidney; 0·1 *M* hydrazine, 1 mg. toxin added solid or in 0·9% NaCl, *p*H 7·4. Fourteen experiments.

		O_2 uptake per hr. (μl.)	
Exp.	Substrate	Control	Toxin
4	*d*-Alanine, 0·1 *M*	95	82
11	*l*-Cysteine, 0·025 *M*	214*	222*
13	*l*-Cysteine, 0·025 *M* (autoxidation—no enzyme)	158	169

* Total O_2 uptake.

Table 8. *Adrenaline oxidase*

Enzyme [Blaschko *et al.* 1937] used crude or purified by precipitation with 30% alcohol or isoelectrically at *p*H 5·0; 0·013 *M* adrenaline, 0·5 mg. toxin, *p*H 7·4. Sixty-two experiments.

			O_2 uptake per hr. (μl.)	
Exp.	Enzyme	Addition	Control	Toxin
35	Crude	2 mg. glutathione	302	303
28	Alcohol ppt.	HCN, 0·001 *M*	35	45
51	Ppt. at *p*H 5·0	0·16 mg. ascorbic acid	174	178

The presence of HCN, glutathione, or ascorbic acid, or of any two of these together, caused no significant alteration in the effect of toxin.

Table 9. *Autoxidation of adrenaline*

Adrenaline 0·006 *M* in 0·1 *M* phosphate (Na and K salts), *p*H 7·4; 1 mg. toxin added in water. Eighty-seven experiments.

		O_2 uptake per hr. (μl.)	
Exp.		Control	Toxin
77		208	211
14	HCN, 0·001 *M*	96	104
42	Normal buffer	308	199
	Na buffer free of K	223	186
49	Normal buffer—toxin in isotonic NaCl	223	164
	K buffer free of Na—toxin in isotonic KCl	121	124

Table 10. *Oxidation of lecithin*

1·6 ml. lecithin suspension (ox liver) in water; toxin and other proteins added solid; glutathione (4 mg. except where otherwise stated) in water in side-bulb, *p*H 4·6. Forty-four experiments.

			O_2 uptake per hr. (μl.)	
Exp.	Toxin (mg.)		Control	Toxin
30	2		105	140
		(Diphtheria antitoxin, 131; toxin—antitoxin (overnight contact), 144 μl. per hr.)		
32	2		143	147
		(Egg-white, 46; wheat-gluten, 110 μl. per hr.)		
36	2		76	100
		(Antitoxin, 89; toxin—antitoxin (mixed direct), 88; cryst. ovalbumin, 88 μl. per hr.)		
33	2		116	177
	1		116	170
	0·5		116	156
	0·25		116	148
41	0·25	Glutathione, 1 mg.	67	79
		Glutathione, 0·5 mg.	73	106

In individual experiments in each group occurred considerable random variations from +50 to −50 %, with and without toxin, but taken collectively the results with toxin showed no real deviation from the normal under most of the conditions studied.

The net O_2 uptake for every enzyme varied with each individual preparation, but duplicate experiments with a fresh preparation showed good agreements in both the control and toxin series; with an uptake of 100 μl. or more per hour and the manometer taps frequently re-greased there was rarely obtained a deviation greater than ±10 %.

With lecithin and glutathione the toxin most frequently caused an acceleration (see Table 10). The accelerations sometimes produced by the other proteins were rarely of the same order as that from toxin.

In the autoxidation experiments with adrenaline where retardations were obtained, the most interesting results were those in absence of either Na^+ or K^+, as shown in Table 9; the retardation in presence of Na^+ and the negative effect with K^+ were too consistent to be due to experimental error. In later experiments, however, with both ions present, consistent retardation was not found, although external conditions were precisely the same.

Phosphorus exchange

Nucleotidase activity. The toxin was tested for nucleotidase activity by incubation for periods of 3 hr. in Krebs flasks with a mixture of nucleotides prepared by hydrolysis of vegetable sodium nucleate with alkali in the cold. Free phosphoric acid was determined colorimetrically before and after incubation, and control vessels were run without toxin. When toluene was present as antiseptic the increase in free phosphoric acid was virtually zero in both vessels.

Phosphorylation. The effect of toxin on three phosphorylation systems was investigated.

For the coupled oxidation-phosphorylation system of Needham & Pillai [1937], the enzyme was prepared from rabbit or guinea-pig skeletal muscle, or fowl's liver brei: the hydrogen acceptor was pyruvate, whilst both glycerophosphates and hexosediphosphate were used as donators. The mixed nucleotides used in the nucleotidase experiments, adenylic acid, or aneurin, served as P acceptor. Experiments were again of the macro-type, the mixtures being incubated for an hour at 38°; inorganic P was determined before and after incubation by the method of Fiske & Subbarow [1925]. Aneurin was found to interfere with the colorimetric determinations by forming a precipitate with the molybdate reagent, and was first removed by adsorption on kaolin.

Total inorganic P disappearance was in general very small, and was in most cases unaltered by toxin even after overnight contact. But with the enzyme suspensions from the muscles of one particular rabbit we obtained a series of results where the toxin caused an increase in P disappearance if left overnight in contact with the enzyme. Table 11 shows typical results from this series. We are inclined to think that these positive results originated in some degree from the particular animal, the treatment of which, however, differed in no way from that of the others except that it was killed by chloroform instead of by a blow on the neck. These active enzyme suspensions were also found to give a precipitin reaction with the toxin.

For investigation of the pyruvate-adenylic acid system of Lipmann [1939] (see Table 5), and the glucose-kidney-extract system of Kalckar [1939], O_2 uptake was measured manometrically and inorganic P afterwards determined in the contents of the flasks. In neither case was the toxin found to affect P esterification.

Table 11. *Phosphorylation of nucleotides*

2 ml. enzyme suspension from muscle of chloroformed rabbit; 0·01 *M* glycerophosphate*, 0·02 *M* pyruvate, 0·01 *M* fluoride, 0·03 *M* phosphate, *p*H 7·4; 5 mg. toxin, overnight with enzyme, 0·5 mg. mixed nucleotides. Total volume, 4 ml. Incubation, 30 min. at 38°.

		Phosphorus (mg.)		
Exp.		Before incubation	After incubation	Esterified
4	Control	5·26	4·65	0·61
	Toxin	5·71	4·17	1·54
8	Control	4·35	4·35	0·0
	Toxin	5·00	4·56	0·44

* B.D.H.—largely β- with some α-isomeride.

Discussion

The results of this investigation up to the point reached in these experiments indicate that the enzyme systems studied are not directly affected *in vitro* by diphtheria toxin. This may be regarded as conclusive in the cases of succinic, lactic, malic and β-hydroxybutyric dehydrogenases, but for the other systems some further investigation to a degree depending on the system concerned might be advantageous.

On the other hand, the symptoms observed in diphtheria which suggested these investigations are still unexplained. The interference with the action of the heart is apparently not the result of inhibition of the muscle oxidases. So far as lactic dehydrogenase is concerned our results form a corollary to those of Corkill & Ochoa [1934], who showed that a toxaemic liver will not permit the resynthesis of glycogen from lactate. It follows therefore that all lactate formed in the body in diphtheria has to be oxidized in the muscles, so that any interference with the action of the lactic dehydrogenase would lead to a pronounced lactacidaemia.

We have so far made no attempt to study the effect of toxin on the oxidation of fats, and it must be remembered that the other symptoms of diphtheria that might be traced back to a biochemical origin—ketonuria and accumulation of fat in the heart—can quite conceivably be the result of interference with these processes.

The 'latent period' between the injection of toxin *in vivo* and the manifestation of its effect constitutes another factor which must be considered in connexion with the chemical effects observed. It is apparently the same length (about 48 hr.) under all conditions. It is known that the toxin on injection passes direct into the muscle tissue, so that one of two processes, or possibly a combination of both, may take place during the latent period. Either the toxin may pass into the interior of the cells and seriously disturb their internal equilibrium by its presence, or a further chemical change of the toxin is necessary before it becomes actually toxic, and occurs in the tissues during the latent period. On the face of it, there is a strong argument against the first possibility in that the appearance of the symptoms is very rapid at the end of the period, whereas if the toxin passed slowly into the tissue it would do its harmful work as it proceeded, so that the symptoms should appear gradually. As regards the second possibility, there is no real proof that the 'toxin' as produced from cultures of *C. diphtheriae* is the actual toxic substance, since it is grown on synthetic media *in vitro*, and has never been isolated from diphtheritic tissue. Further, Pappenheimer [1938] has shown that the toxic properties are destroyed by acetylation with ketene, the number of amino-groups acetylated corresponding closely with the number of ε-amino-groups of lysine in the molecule. He was careful to point out that the toxic properties might equally possibly be due to the spatial arrangement of the ε-amino-groups as to their actual presence, but whichever is the correct postulation the free amino-groups form a basis for further chemical changes to the molecule in the body. If such a reaction were to occur in the body, it is quite conceivable that the 'super-toxin' formed might affect these enzymes in a way which the substance grown *in vitro* would be unable to do.

A serious disadvantage with all these investigations is that up to the present a 'chronic' diphtheritic condition has not been achieved in any animal for *in vivo* study.

SUMMARY

Experiments have been carried out *in vitro* to study the effect of diphtheria toxin on tissue respiration, glycolysis and a number of dehydrogenase and phosphorylation systems under varied conditions.

The dehydrogenase systems for succinic, lactic, malic and β-hydroxybutyric acids have been shown conclusively to be unaffected by toxin, even after preliminary contact between the enzyme and toxin, whilst the indications are that other systems studied are also unaffected. Oxidation of lecithin by glutathione is to some extent accelerated. The effect on autoxidations is inconclusive.

REFERENCES

Blaschko, Richter & Schlossmann (1937). *J. Physiol.* **90**, 1.
Corkill & Ochoa (1934). *J. Physiol.* **82**, 399.
Euler, Albers & Schlenk (1936). *Hoppe-Seyl. Z.* **240**, 113.
Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
Friedemann, Cotonio & Shaffer (1927). *J. biol. Chem.* **73**, 335.
Green (1936, 1). *Biochem. J.* **30**, 629.
—— (1936, 2). *Biochem. J.* **30**, 2095.
—— & Brosteaux (1936). *Biochem. J.* **30**, 1489.
—— Dewan & Leloir (1937). *Biochem. J.* **31**, 934.
Hopkins (1925). *Biochem. J.* **19**, 787.
Kalckar (1939). *Biochem. J.* **33**, 631.
Krebs (1933). *Hoppe-Seyl. Z.* **217**, 351.
—— (1935). *Biochem. J.* **29**, 1620.
—— & Henseleit (1932). *Hoppe-Seyl. Z.* **210**, 33.
Lipmann (1939). *Nature, Lond.*, **143**, 281.
Needham & Pillai (1937). *Biochem. J.* **31**, 1837.
Pappenheimer (1938). *J. biol. Chem.* **125**, 201.
Peters & Cunningham (1937). *Lancet*, i, 375.
Richter (1937). *Biochem. J.* **31**, 2022.
Stotz & Hastings (1937). *J. biol. Chem.* **118**, 479.
Straub (1939). *Biochem. J.* **33**, 787.

23. THE SYSTEM DEHYDROASCORBIC ACID-GLUTATHIONE

BY ERIC MITCHELL CROOK

From The Biochemical Laboratory, Cambridge

(*Received 23 January 1941*)

THE ability of tissue extracts from plants and animals, either to inhibit the oxidation of ascorbic acid (hereafter 'AA'), or to reduce added dehydroascorbic acid ('DHA'), is well known. In his earliest experiments on AA (hexuronic acid), Szent-Györgyi [1928] showed that the peroxidase system of turnip juice would oxidize the natural AA in the juice when H_2O_2 was added, but that ascorbic acid was regenerated on standing. He also found that minced rat kidneys quickly reduced added DHA made by oxidizing AA with I_2, and that this reduction was performed in buffer alone by SH glutathione ('GSH'). Similar results have been obtained by Strachitskii & Rubin [1936], who found that DHA injected into living cabbage leaves was reduced and greatly increased the respiration, and by Goldstein & Volkensohn [1938], who showed that in tumour, placenta, spleen and embryo tissue, the initial oxidation induced by Cu or Fe is followed by reduction back to AA. Also, Golyanitskii & Belonosov [1936] demonstrated that DHA from the oxidation of AA by heat and air is reduced by inclusion in an alcoholic fermentation.

Following Szent-Györgyi's suggestion of the role of GSH, Mawson [1935] explained the protective action of tissue extracts in preventing the destruction of AA partly, at least, by their content of GSH, whilst de Caro & Giani [1934] and Barron *et al.* [1936] showed that GSH was capable of completely protecting AA from oxidation by tissues or by Cu.

Bersin *et al.* [1935], however, were unable to reduce DHA with GSH. Their results are unexplainable and do not tally with those of any other workers. In fact, some of the characteristics of the reaction have been investigated by several workers, notably Borsook *et al.* [1937].

Pfankuch [1934] was the first to describe catalytic reduction of DHA by SH compounds. He showed the presence, in potato juice, of a heat-labile substance, precipitable by protein reagents, which catalysed the formation of AA from DHA and cysteine. Similar findings were reported by Hopkins & Morgan [1936]. GSH was found to prevent the oxidation of AA by the ascorbic oxidase of cauliflower and cabbage juice, but was itself vicariously oxidized in the process. They proved that this was due to a factor in the juice which catalysed the reaction between DHA and GSH, the rate of the uncatalysed reaction being not nearly high enough to account for the protection. Copper was not able to catalyse the reaction, so that its presence in the juice would not account for the effect. Szent-Györgyi [1931] had noticed the vicarious oxidation of GSH in the presence of AA and cabbage juice, but had not recognized its enzymic character. Martin [1940] has recorded a similar vicarious oxidation of 'virus protein' in the presence of AA, which he attributes to the SH groups of the protein and a reaction of the Hopkins-Morgan type.

Reducing catalysts were also demonstrated by Lohman & Sanborn [1937] in the juice of peas and beans, and by Rubin *et al.* [1937] in *Hydrangea, Cyclamen,* *Zea* etc. The former workers have also confirmed Hopkins & Morgan's report that Cu is not responsible.

Fujita & Numata [1938], using pumpkin juice, and Kertesz [1938], using the juice of cauliflowers and cucumbers, were unable to repeat the observations of Hopkins & Morgan, but these have since been confirmed and extended by Crook & Hopkins [1938], who showed the pronounced effect of *p*H and the difference in behaviour between cauliflower and cucumber. The latter contained very little, if any, of the reducing catalyst.

This paper is a preliminary report of the separation of the reducing factor in cauliflower juice from the associated oxidase and the 'resynthesis' of the protection system of Hopkins & Morgan. The work is not nearly complete, but it was felt that what has been done should be put on record as it is not being continued for the time being.

EXPERIMENTAL

Enzyme. The source of enzyme has been the florets of cauliflower and broccoli, both English and foreign. These were minced and pressed and the juice centrifuged, and sometimes fractionated before use. Care was also taken to prevent access of Cu and glass-distilled water used throughout.

Dehydroascorbic Acid. Great difficulty was experienced in evolving a satisfactory and rapid method for making DHA. Attempts to prepare it in the solid state by the method of Moll & Wieters [1936] were not satisfactory. The product was never sufficiently pure and the impurities inhibited the reaction. Unfortunately, there has been no opportunity to try out another method, the details of which were very kindly supplied by Prof. Hirst of Bristol University.

Oxidation of AA by the oxidase plus atmospheric O_2 was used in many cases, as were Cu plus atmospheric O_2, Cu plus H_2O_2, and I_2. The last was also unsatisfactory as the I^- ion inhibits the reducing system very strongly. The most satisfactory method finally evolved was to use a saturated aqueous solution of pure benzoquinone (recryst. from light petroleum B.P. < 40°) in amount just less than the equivalent of the AA so that no quinone remained in excess. Quinone was shown to be a strong inhibitor of the reducing reaction whereas the quinol produced had no such effect.

Reaction velocity. The reactions were followed by two sets of methods. In the earlier experiments, AA was estimated by titration; later by a photoelectric procedure. To follow the course of the oxidation, 10 ml. of solution containing 10 mg. AA, 2 ml. buffer (*M*/5 Sørensen or McIlvaine, *p*H 6) and 1 ml. enzyme solution were shaken on a machine to ensure thorough aeration. Samples were removed at stated intervals, the enzyme destroyed with trichloroacetic acid and AA estimated by titration with *N*/100 I_2. All measurements have been done at *p*H 6 as this value is near the peak of both oxidizing and reducing systems. Reduction of DHA was followed anaerobically as described by Hopkins & Morgan using 2·5 moles of GSH per mole AA (i.e. slight excess of GSH) and estimating AA by the micro-Tillman method [Birch *et al.* 1933; Hopkins & Slater 1935], because of the presence of GSH, which also reacts with I_2. Alternatively, measurements were made aerobically. If DHA had been prepared by shaking with enzyme, no further addition of juice was made. *M*/100 KCN was now used to inhibit the oxidase completely, and, as shown below, this has no effect on the reducing system. Later, all measurements of AA were made by the direct photoelectric method of Evelyn *et al.* [1938], modified for use with larger quantities of ascorbic acid. The AA solution was diluted to contain between 2 and 10 mg. AA per 100 ml. and a strong dye solution—one giving an initial reading of about 18 on the galvanometer scale—was used. For these stronger dye solutions it was necessary to construct a series of calibration curves covering different initial readings, as the straight line relationship breaks down. They are, however, very rapidly and easily made. A Wratten No. 63 filter was found best.

When employing the photoelectric method, the use of trichloroacetic acid for stopping the enzyme action was discontinued, as it was found to interfere with the readings by altering the dye. Instead, the enzyme was destroyed by first making the solution 30 % with respect to acetic acid and later diluting to give the 5 % recommended by Evelyn *et al.*

Again, when DHA was made by oxidizing AA with quinone, the quinol formed rapidly reduced the dye, making titration methods useless. However, the rate of reduction is slow in comparison with that caused by AA. Evelyn *et al.* have shown that the latter is complete within 5 sec. The reduction of dye by AA alone is thus easily found by extrapolation of the galvanometer readings.

Nature of the dehydroascorbic acid

It was at first suspected that the DHA formed by enzymic oxidation of AA differed from that obtained by simple chemical oxidation. GSH, anaerobically and in the presence of the juice, always reduced the latter form at only 60–70 % of the speed at which it reacted with the former. When DHA is prepared by oxidation with a reagent, or by using Cu as catalyst, either the reduced reagent or Cu remains in the solution as an 'impurity' which could conceivably act as an inhibitor. Table 1 shows the average velocity of reduction, by GSH in the presence of the juice, of DHA made by various methods, together with the velocities resulting from the addition of the corresponding 'impurity' to enzymically oxidized AA. Except in the case of large amounts of Cu (which cause precipitation in the juice), these are all higher than the rates with 'chemical DHA'. Thus the greater part of the inhibition is not due to the presence of such substances.

Table 1. *Velocity of reduction of DHA by GSH*

DHA prepared by oxidation with	Reaction rate	Substance added to 'enzymic' DHA	Reaction rate
Enzyme	100	—	—
I_2—I^- ion still present	33	0·04 N I^-	80
I_2—I^- precipitated by Pb	72	0·02 N I^-	89
Cu (20 mg./100 ml.)—H_2O_2	40	—	—
Cu (20 mg./100 ml.)—H_2O_2, Cu removed by dithiocarbamate	61	Cu: 0·2 mg./100 ml.	100
Cu (1 mg./100 ml.)—O_2	66	1·0 mg./100 ml.	94
		2·0 mg./100 ml.	80
		20·0 mg./100 ml.	37
Quinone	66	Quinol: 10 mg./100 ml.	100

In an endeavour to obtain some evidence on the matter, an attempt was made to determine the specific rotation of DHA from each source. This proved very difficult and the results must be regarded with some suspicion, both because the rotation of DHA is very sensitive to changes in pH and concentration and nature of ions, and because periods of more than 20–30 min. enzymic oxidation cause appreciable opening of the lactone ring at the pH used. The short time means that unoxidized AA is present and must be allowed for in calculating the rotation: the juice, too, has a small blank.

The results are set out in Table 2. In Exp. 1, the AA control, the rotation was determined in the presence of NaOH equivalent to the AA, $M/10$ phosphate

Table 2. $[\alpha]_D^{20^\circ}$ *of dehydroascorbic acid*

Exp. no.	Oxidizing agent	No. of repeats	Total specific* rotation	AA not oxidized (%)	Rotation of DHA*
1	Nil	3	23°	100	—
2	I_2	3	54°	0	54° ± 1°
3	I_2	4	39°	0	39° ± 1°
4	I_2	5	32°	0	32° ± 2°
5	Cu-O_2	5	26°	70	33° ± 2°
6	Oxidase	5	25·5°	70	31° ± 2°

* Calculated as AA.

buffer at *p*H 6, and 4 % trichloroacetic acid, to represent the conditions which obtain in the Cu- and enzyme-oxidized solutions after addition of trichloroacetic acid. The figure found, 23° for the D line, compares favourably with that given by Herbert *et al.* [1933] for the Hg yellow line 578 mμ, with pure AA in weakly acid solution. Exp. 2 is included for comparison with the following values, and is pure AA oxidized with I_2 with no additions, i.e. the solution then contains only DHA and HI. The figure again is in good agreement with that of Herbert *et al.* In Exps. 4–6, AA was neutralized with NaOH and the oxidation carried out in phosphate buffer at *p*H 6, during 20 min.; HI, if present, was neutralized by adding the theoretical amount of NaOH; after acidification with 40 % trichloroacetic acid to make the final concentration 4 % the protein coagulum was centrifuged out if present, and the rotation determined. In the experiments with oxidase, a blank was run with the juice and all conditions the same but no AA present. The values shown in the table are corrected for this blank, which amounted to *c.* 20 % of the total rotation. Exp. 3 was carried out as for Exps. 4–6, but the AA was not previously neutralized. It is included to show the effect of the extra amount of Na salts. After oxidation, an aliquot was taken on which unoxidized AA was determined by titration with I_2. The figures for the rotation of DHA in the presence of AA were calculated assuming that no opening of the lactone ring had occurred during the time occupied in oxidation. This is approximately the case, since Borsook *et al.* [1937] have shown that at *p*H 6 and 37°, only 15 % of DHA is hydrolysed in 1 hr. Here the temperature was *c.* 18°, so that the inaccuracy thus introduced would be within the experimental error. For convenience, DHA is calculated as the equivalent amount of AA.

As will be seen, the rotation of the enzymically prepared form does not differ significantly from that of two chemically prepared modifications. We must conclude, therefore, that there is probably no great chemical difference between them.

The possibility of there being some type of loose complex necessary for the activation of DHA, and slowly formed in the juice, was considered, but allowing chemically prepared DHA to stand anaerobically with enzyme for some time before adding GSH was found further to decrease and not to increase the velocity.

The explanation appears to be that an inhibitor is present, and that this is precipitated when AA and the juice are shaken in air to prepare DHA (much protein is precipitated under these conditions). This, of course, does not occur when chemically prepared DHA is used. Weight is added to this conclusion by the finding that heating the juice to 38° for 20–30 min. precipitates a considerable amount of protein and at the same time increases its ability to catalyse the reaction by as much as 20 %.

Separation of the reducing catalyst

It was found to be comparatively easy to obtain the reducing factor virtually free from the associated oxidase, and thus to show that they are distinct entities. Heating to 38–39° for 20–30 min. does not affect the oxidase and actually improves the reducing effect, as has been mentioned. This treatment removes much protein by denaturation and the muddy solution now becomes a clear yellow. On half saturating with ammonium sulphate, approximately 80 % of the oxidase is precipitated and only 30 % of the reducing factor. By raising the concentration of ammonium sulphate to 80 % saturation, the remainder of the 'oxidase' activity is thrown down, together with most of the reducing factor; the total recoveries of both oxidase and reductase being of the order of 90 %. Rapid dialysis of the 50–80 % saturated fraction causes loss of most of the remaining 'oxidase' activity (i.e. it is probably due to Cu^{++} ion), without appreciably lowering the activity of the reductase. Prolonged dialysis, however, affects the latter very considerably, the loss of activity being restored neither by heated juice nor by diffusate from raw juice. This indicates the inactivation of the reducing catalyst rather than the loss of a coenzyme-like compound. Repetition of the precipitation between the 50–80 % saturation limits removes more impurities and again gives a 90 % yield with no oxidase activity.

Properties of the reducing catalyst

The reducing system, either purified or in the plant, is rather labile. When plants are stored in the refrigerator for more than 2 or 3 days, they are generally found to contain little reducing activity. The purified preparation keeps its activity rather better, being reduced to about half in the course of a week. It is more thermolabile than ascorbic oxidase; exposure for half an hour to a temperature of 45° reduces its activity to half, whilst 55° destroys it entirely. On the other hand, 60° has almost no effect on the oxidase.

Extremes of *p*H reduce the activity. Even in buffer at *p*H 7·5 the activity is reduced to one third in 5 hr., and acids precipitate it completely [cf. Pfankuch, 1934].

Table 3. *Effect of inhibitors*

	Reaction velocity	
Final conc. of inhibitor	Reductase	Oxidase
Buffer alone at *p*H 6	100	100
$(NH_4)_2SO_4$: *M*/4	93	—
KI: *M*/25	78	100
M/50	89	100
Cu^{++}: $3 \times 10^{-5}\,M$	100	150
$1{\cdot}5 \times 10^{-4}\,M$	94	—
$3 \times 10^{-4}\,M$	80	—
$3 \times 10^{-3}\,M$*	37	—
Benzoquinone: *M*/1000†	61	—
Quinol: *M*/1000	100	—
M/200	100	—
Dithiocarbamate: *M*/1000	100	80
KCN: *M*/1000	100	0
M/100	100	—
H_2S: *M*/1000	100	0
M/10	110	—
NaF: *M*/1000	86	—
M/100	83	100
Na iodoacetate: *M*/1000	83	100
M/100	30	100

* Some precipitation of protein.

† Added before GSH.

In neither case is the effect reversible. The pH-optimum of 6·7–6·8 given by Crook & Hopkins is confirmed; see Fig. 1. The activity becomes very low below pH 4 and almost impossible to measure above pH 8 because of the rapid hydrolysis of DHA.

The effects of various inhibitors are set out in Table 3, the results for ascorbic oxidase being included for comparison. As will be seen, the reducing catalyst is fairly sensitive to neutral salts; e.g. even moderate concentrations of ammonium sulphate inhibit somewhat, whereas the oxidase is not at all affected. Similarly, quite small concentrations of Cu^{++} ion inhibit a little, although the effect of the strongest concentration shown can be explained, to some extent at least, by the fact that this amount of Cu is sufficient to fix about 40 % of the GSH present, if co-ordinated GSH is assumed to be no longer available for reduction. Such typical inhibitors of the oxidase as KCN and H_2S, which act in $M/1000$ concentration, do not affect the reducing system even at ten times that concentration. In fact the latter speeds up the reaction, apparently being able to act in somewhat the same manner as GSH.

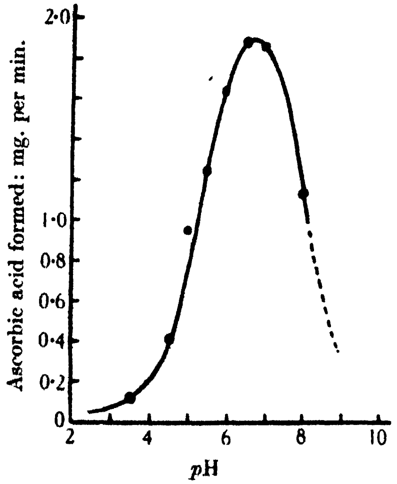


Fig. 1.

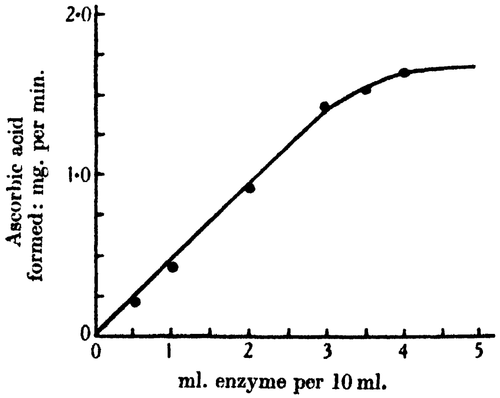


Fig. 2.

Fig. 1. pH-velocity curve for reductase. $M/5$ phosphate-citrate buffer, 1 mg. per ml. dehydroascorbic acid, 4·5 mg. per ml. glutathione (2·5 moles per mole of dehydroascorbic acid), 0·6 mg. per ml. quinol (quinone was used to make dehydroascorbic acid), purified enzyme preparation.

Kinetics

The initial velocity of reaction is proportional to the concentration of catalyst preparation over a wide range, the rate falling off at higher concentrations (Fig. 2—unpurified enzyme). It has not been possible to obtain adequate curves of velocity as a function of concentration of DHA and GSH owing to a shortage of the latter material, but assuming DHA to be the substrate rather than GSH (an assumption only valid for the purpose of this preliminary calculation), the Michaelis constant has been found in a few preliminary experiments to be of the order of $2 \times 10^{-3} M$ for the purified product. This is to be compared with a Michaelis constant for the oxidase of the order of $2 \times 10^{-6} M$.

The straight line kinetics described by Hopkins & Morgan for the reducing mechanism are less evident when purer preparations are used and when the relative concentration of GSH is reduced from 4 moles GSH per mole DHA as used by Hopkins & Morgan [1936] and Crook & Hopkins [1938] to the 2·5 moles per mole here employed. The course of the reaction is initially linear, but when the concentration of GSH is low the curves begin to tail off fairly early (Fig. 3, curve *A*). With 4 moles GSH, this tailing off is hardly perceptible at the concentrations of other reactants here used (Fig. 3, curve *B*). However, when the uncatalysed component of the reaction is allowed for, even 4 moles of GSH do not give a high enough concentration to prevent tailing off. The reaction is obviously extremely complicated, and as its measurement is made more difficult by the simultaneous occurrence of the uncatalysed reaction as well as the hydrolysis of DHA to 2–3-diketogulonic acid, the reasons for this behaviour have not yet been elucidated. Fortunately, the rate of the last-mentioned reaction is sufficiently slow at the *p*H used not to interfere unduly with the significance of the curves given.

Fig. 3, curve *C*, shows a typical curve for the course of the catalysed reaction, this being obtained from curve *A* by subtracting the values of curve *D*, which shows the course of the uncatalysed reaction under the same conditions.

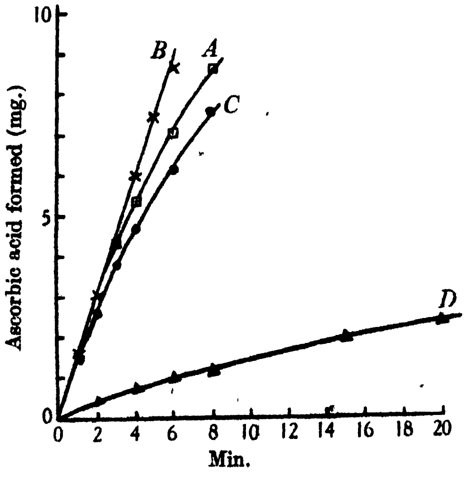


Fig. 3.

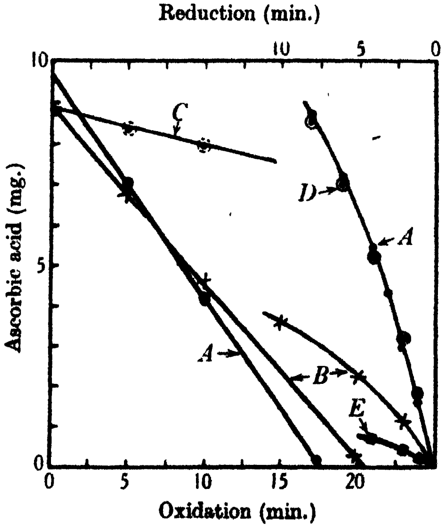


Fig. 4.

The association of this enzyme with ascorbic oxidase in the protective mechanism for ascorbic acid discovered by Hopkins & Morgan was illustrated in two ways. Firstly, by roughly separating the system into two parts by ammonium sulphate fractionation of the juice, testing each separately and then the recombined pair; secondly, by constructing a wholly 'artificial' protection system from oxidase and reducing catalyst.

In the first case, prepared juice was taken and the course of oxidation of AA and of reduction of the DHA thus formed was followed as by Hopkins & Morgan (Fig. 4, curves *A*). A portion of this juice was now separated into 0–50 and 50–80 % saturated fractions with ammonium sulphate. As noted above, the first contains the bulk of the oxidase and only about 20 % of the reducing factor. The course of the curves (*B*) thus obtained show a similar velocity for the oxidase, but a slow reducing reaction. The curve for the uncatalysed reaction (*E*) is included to show that, although the observed reduction of DHA is moderately fast, about half of it is accounted for by the simple chemical reaction. When the

50–80 % fraction is tested, its oxidase content is so low (Fig. 4, curve *C*) that the time required would be so long that much of the DHA would be hydrolysed. Hence this fraction was tested for the reductase in the following way. Oxidation

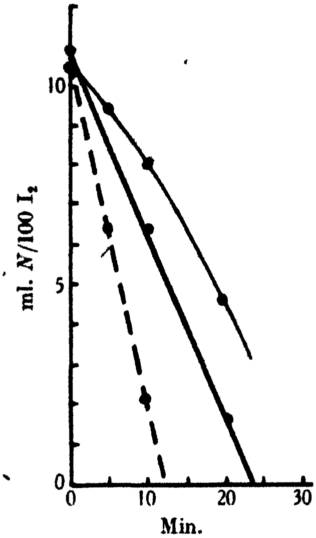


Fig. 5.

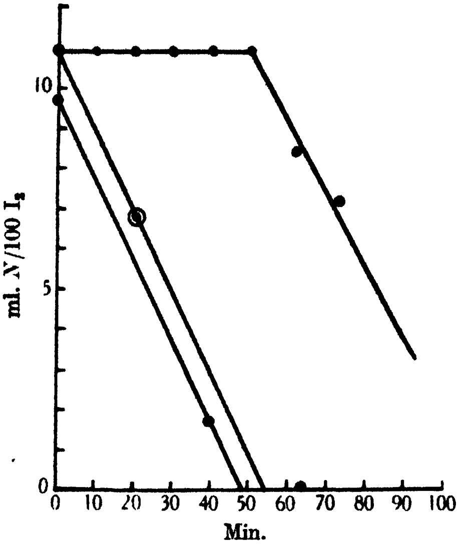


Fig 6.

Fig. 5. Upper curve: ascorbic acid in the presence of glutathione. Middle curve: glutathione. Lower curve: course of the oxidation of ascorbic acid alone.

Fig. 6. Upper curve: ascorbic acid in the presence of glutathione. Middle curve: glutathione. Lower curve: course of the oxidation of ascorbic acid alone.

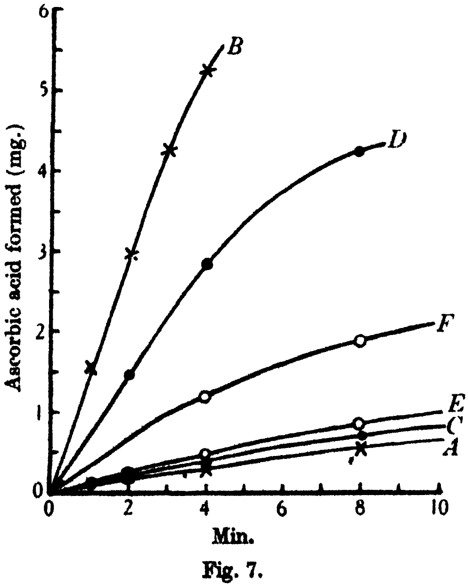


Fig. 7.

of AA was carried out by the oxidase fraction (curve *B*). Now, if the system is reconstitutable, addition of the equivalent amount of 50–80 % fraction should restore the full reducing power of the original juice, which it does (curve *D*, which is indistinguishable from *A*).

In the second set of experiments, cucumber juice was used as a source of the oxidase, as its content of reducing factor has been shown to be very low [Crook & Hopkins, 1938]. The oxidase was further purified by precipitation with 60% saturated ammonium sulphate, re-solution and dialysis. The reductase was purified as above. Concentrations of the reactants are expressed as their equivalents of $N/100\ I_2$ solution, and the experiments were carried out at *p*H 6 as by Hopkins & Morgan. Fig. 5 shows the effect of having much oxidase and relatively little of the reducing factor. The AA is somewhat protected, particularly at the beginning, and the GSH is not oxidized as fast as is ascorbic acid alone. Note too that, as is to be expected, the sum of AA and GSH disappearing in a given time is approximately equal to the amount of AA which disappears in the same time in the absence of GSH. In Fig. 6 the relative concentrations are reversed, there being now relatively more of the reducing system. The result is a typical protection curve, the concentration of AA remaining unchanged until all the GSH has been oxidized at a rate which is the same as that for AA alone.

The behaviour of the reducing factor with cysteine and thiolacetic acid was tested. The courses of the reactions are shown in Fig. 7, *A* and *B* being the chemical and catalysed reactions with GSH, *C* and *D* with cysteine, and *E* and *F* with thiolacetic acid. GSH is obviously far more efficacious than either of the others, whilst thiolacetic acid gives a 'catalysed' reaction which is not much larger than the chemical one.

DISCUSSION

That ascorbic oxidase and the reducing mechanism are separate entities seems to be beyond doubt. The fact that the reducing catalyst can be obtained in good yield virtually free from oxidase properties is the best evidence for this, but almost every other property is different. The Michaelis constant of the oxidase is of the order of a thousand times that of the reductase, the effect of inhibitors quite distinct and the thermolabilities different in the two cases. Finally, their relative activities in different samples of juice differ.

This being the case, it seems worth while to point out that Stewart & Stewart [1936] misquote Hopkins & Morgan when they ascribe the catalytic reduction of DHA to ascorbic *oxidase*, rather than to cauliflower press-juice. Similarly it is not difficult to see why Fujita & Numata [1938] were unable to repeat Hopkins & Morgan's results. They were using an oxidase preparation made according to Ebihara [1939], which would not be expected to contain much reducing catalyst; although they remark, 'if one adds too much oxidase, GSH is also oxidized'! It is surprising that they did not observe oxidation of GSH by DHA even chemically under the conditions used (*p*H 6 and 30°). Tauber [1938] too is wrong in stating that AA is not oxidized in the presence of GSH. The main point is that it can be reduced again faster than it is oxidized; but the O_2 uptake in the system is due to the oxidation of AA and not to the preferential oxidation of GSH.

In this connexion, it is interesting to note that Snow & Zilva [1938] report that dialysed cucumber juice, containing less than 0·1 mg. AA per 100 ml., is capable of oxidizing GSH and cysteine directly, whereas dialysed cauliflower juice is unable to do so unless a trace of ascorbic acid is introduced [Crook & Hopkins, 1938].

It would be as well to consider the evidence as to the enzymic nature of the reducing catalyst, for the activity could well be ascribed to a simple chemical substance acting as a cyclic catalyst, particularly as the increase in reaction velocity is seldom greater than a factor of 7 or 8 (although this, of course, might be explained by its occurrence in very low concentrations). The evidence must be indirect, as no preparation pure enough for chemical investigation has been made. The chief evidence in favour of its being an enzyme is (i) non-diffusibility through a cellophane membrane, (ii) great thermolability, (iii) precipitation by acids and other 'protein reagents' [cf. Pfankuch, 1934], (iv) the occurrence of a *p*H-optimum. The last, rather than a general tendency of the curve, would hardly be expected for a simple substance.

The relation of the reducing mechanism here described to other protective mechanisms for AA which have been reported in plants is not clear. Barron *et al.* [1936] showed that in many plant juices AA remained unoxidized on exposure to air. At the same time they found that GSH protected AA against Cu-catalysed oxidation, a result also found by Hopkins & Morgan. The amount required was very small and equivalent to the Cu present, i.e. GSH acts here by fixing Cu as a non-catalytic complex. Damodaran & Nair [1936] isolated a tannin from the Indian gooseberry (*Phyllanthus emblica*) which would inhibit the oxidation of AA in the press-juice. They concluded that its action depended on suppression of heavy metal catalysis since the protection could be 'over-ridden' by massive amounts of Cu. Kardo-Syssayava & Nissenbaum [1938] report a thermolabile stabilizer for AA in the structural parts of the tomato, but give no indication of the mechanism concerned in its functioning. The same comment is true of the findings of Giri & Krishnamurthy [1940], who were able by treatment with acetone to separate the press-juice of certain plants into a fraction containing ascorbic oxidase and one containing a substance preventing the oxidation of AA, even in the presence of added Cu. Apparently, the effect of this inhibitor was to depress the O_2 uptake of the system generally, so that they are probably not dealing with the Hopkins-Morgan system. The claim of Giri & Krishnamurthy that, previous to their publication, there has been no indication in the literature of the coexistence of oxidizing and protective factors for AA in plants is hardly substantiated by the literature reviewed in this paper.

The possibility that the Hopkins-Morgan system or a modification thereof is one plant analogue of the cytochrome system in animals is probably worth considering. While not wishing to suggest that it is so, it may be worth while setting out certain observations in its favour. Several authors, notably Ganapathy [1938] and Lohman & Sanborn [1937], have shown that —S—S— is reduced in plant juices. The former has also shown that this effect is abolished by heating, i.e. depends on further enzyme systems. Further, Povolockaja [1937] found that the O_2 consumption of the AA ascorbic oxidase system fully accounts for that of the whole germinating seed in legumes and cereals. Thus the Hopkins-Morgan system could conceivably act as the last link in a chain:

$$\text{H} \xrightarrow[\text{plant substrates}]{} \underbrace{\text{—S—S—} \xrightarrow[\text{reductase}]{} \text{DHA} \xrightarrow[\text{ascorbic oxidase}]{} \text{atmospheric } O_2}_{\text{Hopkins-Morgan system}}$$

The criticism by Barron [1939] of the validity of the work of Hopkins & Morgan and Crook & Hopkins on the grounds that both the ratio AA : GSH and the total concentrations used were unphysiological loses much of its weight in the light of the above considerations. In fact the concentrations of AA used by Hopkins and his collaborators are well within the ranges exhibited by plants and are much below that occurring in such plants as the black currant and Hungarian red pepper. Neither were their ratios unphysiological, since, according to the work of Pett [1936], GSH and AA occur in potatoes in a molar ratio of approximately 2 : 1.

More recent work, such as that of Ganapathy & Sastri [1938], who showed that papaya and similar fruits contain as much as 0·3 % of GSH, casts further doubt upon the validity of Barron's sweeping statement that 'there is very little glutathione in plants and a large amount of ascorbic acid'.

SUMMARY

1. The enzyme catalysing the reduction of dehydroascorbic acid by SH—glutathione has been separated from the associated ascorbic acid oxidase.
2. It is shown to be responsible for the protection of ascorbic acid by glutathione in cauliflower juice.
3. The fact that its activity varies in different plants has been mentioned.
4. Its properties and some preliminary observations on the kinetics of the reaction are described.
5. Its possible role as an 'end system' in plants is discussed.
6. Some indication is given that oxidation of ascorbic acid by the oxidase or by simple chemical reagents gives rise to the same form of dehydroascorbic acid.

The author wishes to express his indebtedness to Prof. Sir Frederick Gowland Hopkins for his continued helpful interest and gracious encouragement. He also wishes to thank Melbourne University for the award of the Australian Commonwealth Travelling Scholarship, during the tenure of which the experimental work in connexion with this and the previous paper was carried out.

REFERENCES

Barron (1939). *Cold Spr. Harb. Symp.* **7**, 145, 146.
—— Barron & Klemperer (1936). *J. biol. Chem.* **116**, 563.
Bersin, Köster & Jusatz (1935). *Hoppe-Seyl. Z.* **235**, 12.
Birch, Harris & Ray (1933). *Biochem. J.* **27**, 590.
Borsook, Davenport, Jeffreys & Warner (1937). *J. biol. Chem.* **117**, 237, 245, 269.
de Caro & Giani (1934). *Hoppe-Seyl. Z.* **228**, 13.
Crook & Hopkins (1938). *Biochem. J.* **32**, 1356.
Damodaran & Nair (1936). *Biochem. J.* **30**, 1014.
Ebihara (1939). *J. Biochem., Tokyo*, **29**, 199.
Evelyn, Malloy & Rosen (1938). *J. biol. Chem.* **126**, 645.
Fujita & Numata (1938). *Biochem. Z.* **299**, 249.
Ganapathy (1938). *Curr. Sci.* **6**, 451.
—— & Sastri (1938). *Proc. Ind. Acad. Sci.* B, **8**, 399.
Giri & Krishnamurthy (1940). *Nature, Lond.*, **146**, 99.
Goldstein & Volkensohn (1938). *Biokhimia*, **3**, 446.
Golyanitskii & Belonosov (1936). *C.R. Acad. Sci. U.R.S.S.* (N.S.), **4**, 15.
Gudlet & Kardo-Syssayava (1938). *Biokhimia*, **3**, 334.
Herbert, Hirst, Percival, Reynolds & Smith (1933). *J. chem. Soc.* p. 1270.
Hopkins & Slater (1935). *Biochem. J.* **29**, 2803.
—— & Morgan (1936). *Biochem. J.* **30**, 1446.
Kardo-Syssayava & Nissenbaum (1938). *Biokhimia*, **3**, 354.
Kertesz (1938). *Biochem. J.* **32**, 621.
Lohman & Sanborn (1937). *J. Industr. Engng Chem.* **20**, 189, 1195.
Martin (1940). *Rep. 3rd Int. Congr. Microbiol.* p. 281.
Mawson (1935). *Biochem. J.* **29**, 569.
Moll & Wieters (1936). *Merck's Jber.* **50**, 65.
Pett (1936). *Biochem. J.* **30**, 1228.
Pfankuch (1934). *Naturwissenschaften*, **22**, 821.
Povolockaja (1937). *C.R. Acad. Sci. U.R.S.S.* **17**, 35.
Rubin, Sesakian & Lulikova (1937). *C.R. Acad. Sci. U.R.S.S.* **15**, 495.
Snow & Zilva (1938). *Biochem. J.* **32**, 1926.
Stewart & Stewart (1936). *Ann. Rep. Chem. Soc.* **33**, 389.
Strachitskii & Rubin (1936). *Biokhimia*, **1**, 642.
Szent-Györgyi (1928). *Biochem. J.* **22**, 1387.
—— (1931). *J. biol. Chem.* **90**, 385.
Tauber (1938). *Ergebn. Enzymforsch.* **7**, 308.

24. A NEW METHOD FOR THE DETERMINATION OF PHYTOHORMONE ACTIVITY

By GEORGE EDGAR TURFITT

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine

(*Received 26 November 1940*)

In view of the widespread occurrence of phytohormones amongst the bacteria, yeasts and fungi, it would not be surprising to find that growth promotion in these micro-organisms is governed by a mechanism fundamentally similar to that obtaining in the higher plants. No evidence has been obtained [Boysen Jensen, 1932; Nielsen & Hartelius, 1932; Bonner, 1932; Bunning, 1934], however, that the phytohormones produced by micro-organisms play any role in their growth. Stimulation of a metabolic activity has, on the other hand, been recorded by Grace [1937], who found that in the fermentation of sugar solutions by baker's yeast, gas production (measured in a saccharimeter after 1 hour) was greatly accelerated as compared with the control by 1 part per million of α-naphthylacetic acid, and to a lesser extent by 5 p.p.m. In other experiments, $\frac{1}{10}$ p.p.m. gave considerable stimulation, and as little as $\frac{1}{250}$ p.p.m. had some effect. Concentrations higher than 5 p.p.m. had a repressing action. Similar responses were obtained with β-indolylacetic acid, its propionic and butyric homologues, and with the K salt of α-naphthylacetic acid, all of which substances are known to possess a stimulating effect on plant growth.

The adoption of some standardized procedure based upon the growth of yeast, or upon the stimulation of its fermentative power, would provide a new and eminently satisfactory method for the rapid testing of materials suspected of phytohormone activity. The work described in this paper was undertaken in order to ascertain the possibility of devising a sorting test along these lines.

EXPERIMENTAL

The yeast used throughout the work was an English top fermentation type ('D.C.L.' brand), and a separate sample was obtained fresh from the manufacturers for each experiment.

1. *Stimulation of fermentative activity*

A. *Grace's technique.* The exact conditions under which Grace carried out his experiments are not described in detail in his paper, but an attempt has been made to repeat the work under conditions as nearly identical as the description permitted. The saccharimeter technique possessed obvious inherent defects, and seemed fundamentally unsound especially in that no control could be exercised over the volumes of gas lost through the open limb. As a consequence of uneven and varying sedimentation of the yeast cells, the volumes of gas issuing up either limb showed wide variations in control experiments, the results of which were never sufficiently reproducible to warrant the use of the method for the testing of hormone solutions.

B. *Eudiometer technique.* This procedure eliminates the most serious defects of the saccharimeter method, and on theoretical grounds information of a more accurate nature should be obtained by its use. A wide range of experiments has been carried out in order to determine the conditions under which the stimulating effect was most pronounced.

(1) The following were placed in a round-bottom 50 ml. flask:

10 ml. water,[1]
10 ml. 10 % sucrose solution,
4 ml. yeast suspension (5 g. yeast/20 ml. water),
1 ml. hormone solution (replaced by water in control experiments),

the flask being then closed with a rubber bung carrying a delivery tube. Five flasks were prepared in this way, giving the following hormone concentrations: 0 (control), $\frac{1}{10}$ p.p.m., 1 p.p.m., 5 p.p.m., 10 p.p.m. The flasks were placed in a water bath at a constant temperature of 24°, and the CO_2 produced during the fermentation was collected in eudiometer tubes over saturated brine, the volumes being noted at intervals. After 24 hr. the reaction was very slow; each flask was shaken until no further bubbles of gas were evolved. Several series of control experiments (in which the 1 ml. hormone solution was replaced by 1 ml. water) were carried out in this way, whilst both β-indolylacetic acid and α-naphthylacetic acid were examined using the same yeast sample. Representative figures are quoted in Table 1.

Table 1

Figures are ml. CO_2 evolved.

Concentrations	Control experiments					β-Indolylacetic acid					α-Naphthylacetic acid				
(p.p.m.) ...	0	0	0	0	0	0	$\frac{1}{10}$	1	5	10	0	$\frac{1}{10}$	1	5	10
Time (hr.)															
1	14·5	10·5	15	13·5	14	11·5	15·5	12·5	11·5	10·5	7	8·5	10·5	8	8
2	45	35·5	47	40	47	35	42	36	33	29·5	28·5	30	35	27	23
3	74	60	75	72	78	60	67	62	58	56	58	53	59·5	56	49
4	98	77	93	98	104	78	86	80	76	73	77	78	83	74	70
5	112	94	112	117	124	92	100	97	90	90	93	99	105	94	81
6	123	107	126	131	136	103	113	108	102	100	—	—	—	—	—
24	173	169	168	169	170	166	177	177	178	173	178	176	179	180	177
24 (after shaking)	182	180	179	180	182	183	185	188	184	182	184	186	189	185	180

From these experiments it would appear that some stimulation does occur as a result of the presence of hormone, the optimum effect being with a concentration of 1 p.p.m., and that with concentrations of more than 5 p.p.m. there is a repressing effect. Neither stimulation nor repression, however, is very marked.

(2) The bios complex, normally responsible for growth promotion in yeasts, is to some extent synthesized by the organism itself. Thus there exists within the cells a quantity of growth stimulant varying in amount in different samples. In an endeavour to obtain the yeast free from bios and related substances, the yeast samples were washed with 0·9 % NaCl prior to use. The washing was repeated three times in each case, and the experiment then carried out as described under (1) above. Under these conditions neither stimulation nor

---

[1] An aqueous mineral salt solution of the following composition: $(NH_4)_2SO_4$ 0·2 %, KH_2PO_4 0·2 %, $MgSO_4,7H_2O$ 0·05 %, $FeSO_4,7H_2O$ 0·001 %, has been substituted in several experiments, but without any appreciable difference in the results.

repression was exhibited. Although the matter was not further investigated, this negative result with washed yeast tends to suggest that the promotion of fermentation may be dependent upon the presence of a system of substances whose activities are in some way closely inter-related. A similar conclusion has been reached by Kögl [1935] on the functions of the separate fractions of the bios complex in relation to yeast growth.

(3) The precise influence of O_2 on the reaction was investigated by carrying out the fermentation of a series of controls under both aerobic and anaerobic conditions.

In the aerobic experiments, individual charges, each in a 60 ml. flask, were composed of:

20 ml. water,
20 ml. 10 % sucrose solution,
8 ml. yeast suspension,
2 ml. water (replacing hormone solution in controls).

20 g. yeast were suspended in water to a total volume of 80 ml. This was aerated with a stream of sterile air during 2½ hr., when 8 ml. of the suspension were used for each experiment. Figures for a representative batch of five flasks are given in Table 2.

Table 2. *Control experiments*

Figures are ml. CO_2 evolved

Time (hr.)	Aerobic technique					Anaerobic technique				
1	8	9	8·5	9	8	12	11	12	10·5	11
2	52	51·5	53	48	51	47	48	48·5	46·5	47
3	88	91·5	89·5	90	88·5	96·5	98	98	97	98·5
4	118	123	131	117	133	147	148	148	147	149

For the anaerobic series, charges of similar composition were prepared, but instead of aerating the yeast suspension it was treated with a stream of sterile N_2 during 2½ hr., after which 8 ml. were transferred under strictly anaerobic conditions to each of the rest of the charges. For the purpose of this transfer, the apparatus shown in Fig. 1 was constructed. 20 g. yeast were thoroughly ground up with boiled water to give a total volume of 80 ml. suspension, which was introduced into flask *A*. A stream of N_2 from a cylinder, freed from O_2 by a series of alkaline pyrogallol washings, was bubbled through the whole apparatus during a period of 2½ hr., clips *B* and *C* being open. Meanwhile, the ten 60 ml. flasks, each containing 20 ml. water and 20 ml. 10 % sucrose solution, had been heated to boiling for 2 min. to expel air, re-entry of the latter being prevented by Bunsen valves. The flask *A* was inverted, and the contents transferred to the graduated burette by N_2 pressure. The wash bottle *X* was reversed, the clips being closed during the process to prevent the entrance of air. Each flask in turn was attached to the burette (*Y* to *Z*), and 8 ml. yeast suspension introduced. The process to this point was strictly anaerobic, but the anaerobic introduction of hormone solution presents difficulty. The method adopted consisted in preparing the hormone solutions with freshly boiled water, and delivering the required 2 ml. by means of a pipette attached to an alkaline pyrogallol wash bottle. The flasks were then incubated in a water bath at 25° for 2 hr., the Bunsen valves being removed under brine, and the CO_2 evolved collected in eudiometer tubes. Typical results are shown in Table 2.

(4) The anaerobic technique was undoubtedly the most satisfactory of the various methods investigated, and was employed in the testing of the stimulating

power of a number of substances. The figures given in Table 3 are representative of those obtained in a wide range of experiments.

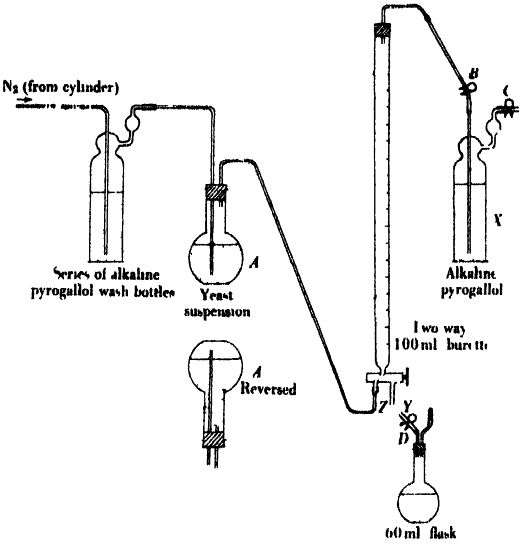


Fig. 1.

In the case of these four compounds both stimulating and repressing effects are obtained. With anthraquinone-1:5-disulphonic acid and anthraquinone-2-sulphonic acid, on the other hand, there is no appreciable effect in either direction.

Table 3

Figures are ml. CO_2 evolved

Concentration (p.p.m.) ...	β-Indolylacetic acid					α-Naphthylacetic acid				
Time (hr.)	0	$\frac{1}{10}$	1	5	10	0	$\frac{1}{10}$	1	5	10
1	14·5	15	15·5	13·5	14	12·5	13·5	17	14	12·5
2	40·5	42	41	43	43	41	43	45·5	41·5	41
3	85	87	92	88	87·5	82·5	87	92	85	85
4	123	132·5	137	138	129	126	130	141	140	139

Concentration (p.p.m.) ...	Phenylacetic acid					β-Naphthylacetic acid				
Time (hr.)	0	$\frac{1}{10}$	1	5	10	0	$\frac{1}{10}$	1	5	10
1	10·5	10	10·5	9·5	9·5	10	9·5	10·5	10	9
2	29	29·5	29·5	27·5	28	28·5	28	30	27	29
3	48	51	52	50	50·5	45	46·5	50·5	46	48·5
4	69·5	79	80·5	78	71·5	67·5	72	77·5	70	71
5	88·5	99	100·5	90	91·5	89·5	95	98·5	98·5	97
6	102·5	118	122	110	112	101	112·5	117	111	109
24	179	181	189	183	172	177	179	188	180	177·5

2. *Stimulation of growth*

In an endeavour to obtain optimum and, so far as possible, standardized experimental conditions, a wide range of influencing factors was examined:

I. Culture medium: (*a*) qualitative and quantitative composition, (*b*) character of the C source, (*c*) character of the N source, (*d*) *p*H, (*e*) influence of 'growth factors'.

II. External conditions: (*a*) temperature, (*b*) duration of experiment, (*c*) effect of O_2.

III. Inoculum: (*a*) biological nature of yeast, (*b*) physiological condition, (*c*) initial concentration.

The extensive work of Thorne [1933, 1; 1933, 2; 1936] in this field has been followed and, where necessary, supplemented along the lines required by the present problem.

A preliminary experiment was carried out in order to gain some idea of the accuracy of the direct cell-counting method of estimation of the yeast suspension. This is recorded in some detail since an identical experimental technique was adopted in later work with hormone solutions. 100 ml. of the culture medium:

KH_2PO_4	2 g.	Brought to *p*H 4·2 by addition of H_2SO_4
$MgSO_4, 7H_2O$	1 g.	
$CaSO_4$	0·2 g.	
$FeSO_4, 7H_2O$	0·1 g.	
$(NH_4)_2HPO_4$	2·5 g.	
Sucrose	25 g.	
Distilled water to	1000 ml.	

were placed in each of three 250 ml. conical flasks and sterilized by steaming during 1 hr. on each of three successive days. To each flask was added 1·0 ml. of a yeast suspension containing 1–2 g. yeast per 100 ml.; the seeding rate (i.e. initial count in the culture medium) was thus obtained as approximately 1–3 million cells per ml. The flasks were incubated at 25°, and counts were taken after three days. For the counts a Thoma-Zeiss haemocytometer was employed, all pronounced yeast 'buds' being arbitrarily counted as individual cells.

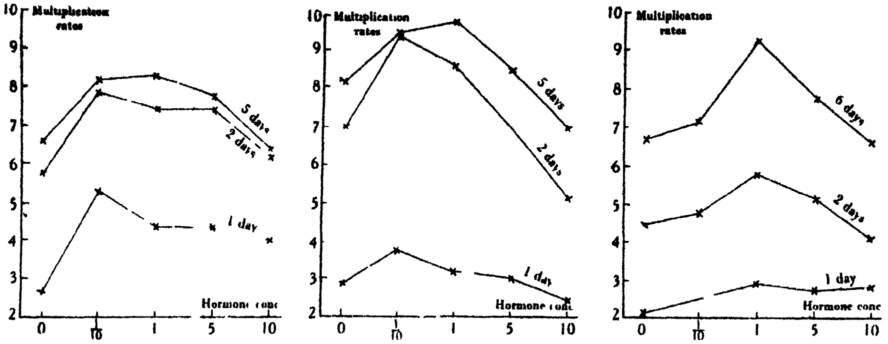
	Exp. 1	Exp. 2	Exp. 3
Initial count	117	89	97
	118	97	101
	113	99	99
	118	107	95
	126	91	98
	1,184,000	970,000	980,000
3 day count	633	399	589
	635	533	659
	604	648	431
	666	580	542
	6,360,000	5,400,000	5,500,000

For comparative purposes these figures may conveniently be expressed as 'multiplication rates', i.e. outcrop rate/seeding rate:

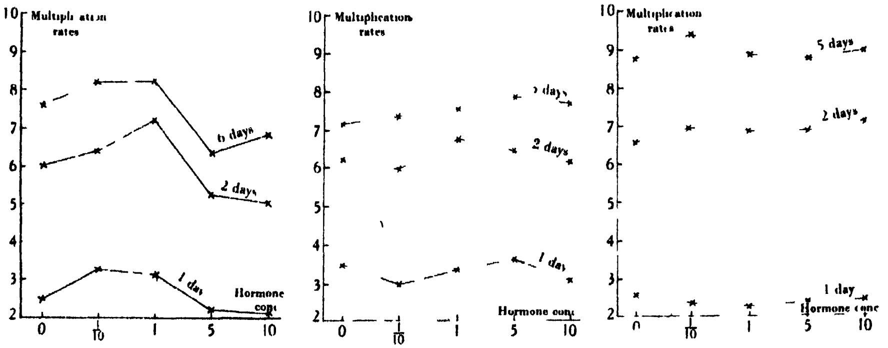
(1) 5·4, (2) 5·6, (3) 5·6.

The degree of accuracy shown in these preliminary controls was thus of a high order, and the investigation was extended to the use of known plant stimulants, and also to compounds hitherto not examined in this respect. In each case the following procedure was adopted.

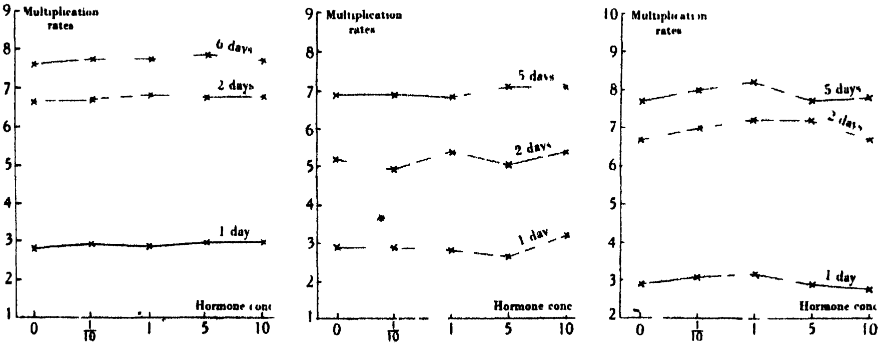
Five 250 ml. conical flasks, each containing 95 ml. synthetic medium were sterilized by steaming. Hormone solutions of different concentrations (which had been prepared with freshly boiled water in order to obtain them as nearly sterile as possible without applying heat) were added in 4 ml. quantities, so that the final concentrations in the media were 0 (4 ml. sterile water added), $\frac{1}{10}$, 1, 5, 10 p.p.m. respectively. Finally, 1 ml. yeast suspension was added to each flask. After taking the initial counts, the cultures were incubated (water



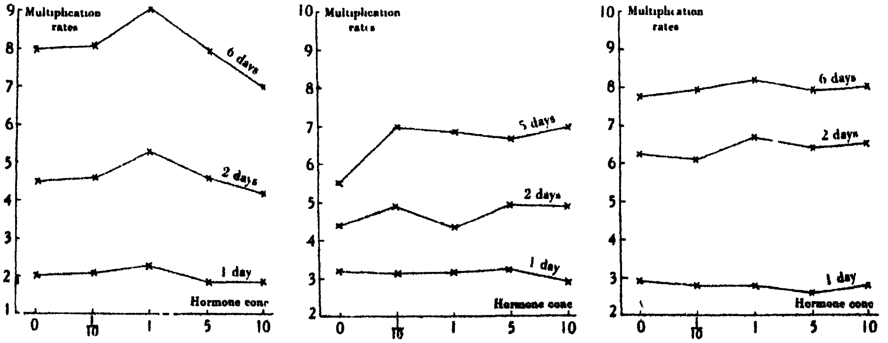
β-Indolylacetic acid α-Naphthylacetic acid Phenylacetic acid



β-Naphthylacetic acid α-Anilinopropionic acid α-Anilinovaleric acid



Anthraquinone-2-sulphonic acid Na anthracene-9:10-*endo*-αβ-succinate α-Phenylpropionic acid



α-Phenylcrotonic acid Glycollic anilide Phenylglycine

Fig. 2. Hormone concentration given in p.p.m.

bath) at 25°, and further counts performed after 1, 2 and 5 (or 6) days. Interpretation and comparison of the results obtained with the test substances are simplified by the graphical representations in Fig. 2.

In order to indicate the differences to be expected between inactive materials and those having pronounced or weakly stimulating effects, the results obtained with two such inactive substances (viz. anthraquinone-2-sulphonic acid, Na anthracene-9:10-*endo*-αβ-succinate) have been included.

DISCUSSION

In an endeavour to obtain comparative data on the fermentation-stimulating activities of various substances, a wide range of experimental conditions has been investigated. The results have served to substantiate the main conclusions of Grace [1937], but the striking stimulating and repressing effects recorded by him have not been confirmed. Since it has been generally accepted that the auxins produced by yeasts and other micro-organisms have no effect on their growth, this stimulation of a metabolic activity of yeast is of considerable interest. The mechanism whereby the phytohormone action is exerted is not clear, but as preliminary washing of the yeast samples before use for fermentation nullifies the stimulating and repressing effect of these substances, it must be concluded that their action is linked with other factors which are removed or deranged by the washing process. Without further investigation of this point it would be premature to discuss the nature of the other factors involved. Went & Thimann [1937] have shown that a close inter-relation exists between β-indolylacetic acid, biotin and sucrose in their promotion of root formation on etiolated pea stems. Even the individual members of the bios complex are known [Kögl, 1935], as mentioned above, to be mutually interdependent for the optimum stimulation of yeast growth. This concept of a system of substances with interlocking functions is now established in many natural processes, and the possibility of a phytohormone-bios system cannot be overlooked in connexion with the stimulation of yeast fermentation.

Although fermentation has proved unsatisfactory as the basis of a sorting test for phytohormone activity, a standardized experimental procedure has been evolved, based upon the stimulation of yeast growth. The somewhat lengthy process of testing stimulating power against the higher plants is thus reduced to the few hours required for the reproduction of yeast cells.

SUMMARY

1. Fermentation of yeast has been shown to be mildly stimulated by the presence of minute quantities of the known phytohormones, β-indolylacetic acid, α-naphthylacetic acid, β-naphthylacetic acid, phenylacetic acid.

2. It has not been found possible to standardize experimental conditions sufficiently to justify the use of the method as a sorting test for substances suspected of phytohormone activity.

3. The effect of washing yeast samples before fermentation is to nullify the stimulating effects ordinarily obtained with phytohormones. This is suggested as tentative evidence for the assumption of a phytohormone-bios system, the action of individual members of which is closely interdependent.

4. Contrary to accepted opinion, evidence has been obtained of the stimulation of yeast growth by phytohormones, and several compounds have been tested for phytohormone activity by observing their effects on yeast growth.

The author wishes to express his thanks to Prof. H. Raistrick in whose Laboratories this work has been carried out, and to Imperial Chemical Industries (Dyestuffs Group), Ltd., for their kind permission to publish the results given in this paper.

REFERENCES

Bonner (1932). *Biol. Zbl.* **52**, 565.
Boysen Jensen (1932). *Biochem. Z.* **250**, 270.
Bünning (1934). *Ber. dtsch. bot. Ges.* **52**, 423.
Grace (1937). *Canad. J. Res.* **15**, 544.
Kögl (1935). *Ber. dtsch. chem. Ges.* **68**, 16.
Nielsen & Hartelius (1932). *C.R. Lab. Carlsberg*, **19** (8), 1.
Thorne (1933, 1). *J. Inst. Brew.* 597.
—— (1933, 2). *J. Inst. Brew.* 608.
—— (1936). *J. Inst. Brew.* 15.
Went & Thimann (1937). Phytohormones. New York.

25. THE CONVERSION OF LAEVULOSE AND FATTY ACID INTO GLUCOSE

BY CORBET PAGE STEWART AND JAMES CARRUTHERS THOMPSON

From the Clinical Laboratory, Royal Infirmary, Edinburgh

(*Received 9 December 1940*)

IT has often been suggested that laevulose is converted into glucose in the animal body and a good deal of evidence has accumulated to support this view. Most of the evidence is indirect, but Cori & Shine [1936] claimed, by incubating liver slices with Ringer's solution, to have demonstrated the direct conversion of laevulose into glucose: they gave no experimental details. Goda [1937; 1938] showed that laevulose disappeared when incubated with liver or kidney slices suspended in Ringer's solution. In an attempt to prove more conclusively the direct conversion of laevulose into glucose, tissue slice experiments have been carried out and a complete carbohydrate balance sheet drawn up.

The possibility of glucose formation from laevulose was first shown by Reilly *et al.* [1898] who used phlorhizinized dogs. Mann & Magath [1922] found that laevulose was effective in hepatectomized dogs only if given some time before hypoglycaemic convulsions developed and suggested that glucose formation must be slow. Later, Bollman & Mann [1931] showed that such dogs could be kept alive for many hours without glucose if laevulose was given in sufficiently large amounts before the blood sugar was greatly lowered. It has been shown by Noble & Macleod [1924], Herring *et al.* [1924] and Corley [1929] that laevulose is not nearly so effective an antidote for preventing shock following the injection of insulin as glucose, unless it is given very soon after the insulin. Immediate protection would, it is presumed, have been afforded in all cases if laevulose could effectively replace glucose without being first converted into glucose or glycogen.

That the liver is the main site of the conversion is indicated by the work of Isaac [1920], Bodansky [1923], Lampson & Wing [1926], Corley [1929] and Mann & Magath [1922]. It has, however, been suggested that other tissues may have the ability to convert laevulose into glucose. Bollman & Mann [1931] showed that whilst laevulose was effective in preventing hypoglycaemic convulsions in hepatectomized dogs if given early enough, it was ineffective in eviscerated hepatectomized dogs, from which it was concluded that the intestines are a second site for the transformation. This is supported by Verzár & McDougal [1936] and Laszt [quoted by Verzár & McDougal, 1936] who consider that laevulose is converted into glucose in the intestine. These results have been contraverted by Steinberg [1927], Oppel [1929], Burget *et al.* [1932] and by Griffith & Waters [1936] who found that laevulose is utilized and can prolong life in dogs deprived of their liver and viscera. Goda [1938] showed that laevulose disappeared and glucose concentration was increased when laevulose was incubated with kidney slices. Steinberg [1927] showed that the isolated surviving skeletal muscle was able to utilize laevulose directly as efficiently as it could utilize glucose. This result supports McGuican [1908] and is confirmed by Griesbach [1929], Bornstein & Völker [1929] and Okamura [1938]. Griffith & Waters [1936] also suggest that laevulose is utilized by the muscles and brain because it prolongs the life of eviscerated dogs. On the other hand, Maclean & Smedley [1912] and Stewart & Gaddie [1934] were unable to demonstrate any utilization of laevulose by the heart muscle of the dog, rabbit or frog, whilst a similar negative result was obtained by Ashford [1933] for brain tissue.

EXPERIMENTAL

The R.Q., O_2 uptake and lactic acid formation of freshly sliced tissue were determined by the method of Dickens & Šimer [1931], 100 mg. moist tissue slices (dried between filter papers) and 1·5 ml. of physiological salt solution [Krebs & Henseleit, 1932] to which laevulose was added, being used for each experiment. Total sugar and laevulose were determined before and after respiration by the method of Hagedorn & Jensen and a modification of that of Herbert [1938] respectively, whilst total carbohydrate was determined by the method of Ochoa [1930]. The respiration time in every case was 2 hr. The carbohydrate oxidized was calculated from the R.Q. and the O_2 uptake. Liver, kidney, brain, skeletal and cardiac muscle, spleen, and intestinal muscle and mucosa of the rat were the tissues examined. A typical balance sheet for liver slices is shown in Table 1.

Table 1. *Balance sheet for liver slices*

O_2 uptake	426 µl.
R.Q.	0·78
CO_2 equivalent of lactic acid formed	146 µl.

	Before (mg.)	After (mg.)
Laevulose	2·33	1·26
Total sugar of medium (as glucose)	3·47	3·92
Total carbohydrate of tissue (as glucose)	1·25	0·45
Glucose converted into lactic acid	—	0·59
Carbohydrate oxidized (as glucose)	—	0·12
Total carbohydrate accounted for	4·72	5·08
Laevulose disappeared	1·05 mg.	

Table 2 shows the results obtained with the various tissue slices. In the first experiments, in which laevulose was the only sugar present in the medium, a big leakage of carbohydrate from the tissue into the medium occurred. This was common to all experiments in which the medium did not contain glucose and so in the subsequent experiments glucose as well as laevulose was present in the medium.

These experiments clearly show the direct conversion of laevulose into glucose by the liver and the kidney, since the amount of laevulose which disappeared is greater than the sum of the carbohydrate oxidized and that converted into lactic acid. All the remaining tissues examined failed to bring about the conversion, the amounts of laevulose which disappeared being negligible. This is especially interesting in the case of the intestine, since it contraverts the conclusions, from indirect evidence, of Bollman & Mann [1931], Verzár & McDougal [1936] and Laszt [quoted by Verzár & McDougal, 1936]. That the conversion is an aerobic reaction is shown by Exp. 14, in which liver slices were incubated with the usual laevulose-containing medium in an atmosphere of N_2 without the disappearance of any laevulose. It may be mentioned here that many attempts have been made, without success, to prepare an active liver brei which would bring about the conversion of laevulose into glucose (or glycogen). No stress can be laid on the R.Q. in the experiments with muscle and mucosa, since the

Table 2

Exp.	O_2 uptake µl.	R.Q.	µl. CO_2 equiv. of lactic acid formed		Laevulose mg.	Total sugar (medium) mg.	Total carbohydrate (tissue) mg.	Glucose converted into lactic acid mg.	Carbohydrate oxidized mg.	Total carbohydrate accounted for mg.	Laevulose disappeared mg.
						Liver					
6	357	0·87	85	B	2·53	4·35	0·63	—	—	4·98	—
				A	1·28	4·25	0·85	0·34	0·24	5·68	1·25
					(8 other exps. gave closely similar results)						
						Kidney					
13	740	0·81	68	B	2·08	3·42	0·68	—	—	4·10	—
				A	1·14	2·80	0·55	0·28	0·31	3·94	0·94
					(3 other exps. gave closely similar results)						
						Liver in an atmosphere of N_2					
14	−24	—	109	B	3·02	3·78	0·40	—	—	4·18	—
				A	2·92	3·78	0·45	0·44	Nil	4·67	0·10
					(1 other exp. gave a closely similar result)						
						Brain					
16	236	1·09	28	B	2·74	3·55	0·20	—	—	3·75	—
				A	2·69	3·20	Nil	0·11	0·29	3·60	0·05
					(2 other exps. gave closely similar results)						
						Skeletal muscle					
20	44	(1·56)	Nil	B	2·75	3·95	0·62	—	—	4·57	—
				A	2·75	4·10	0·67	Nil	0·05	4·82	Nil
					(1 other exp. gave a closely similar result)						
						Duodenum (muscle and mucosa)					
22	157	1·07	125	B	2·53	3·37	0·80	—	—	4·17	—
				A	2·42	3·05	0·50	0·50	0·19	4·26	0·11
					(1 other exp. gave a closely similar result)						
						Duodenum (mucosa)					
25	60	(0·42)	100	B	3·30	3·92	0·23	—	—	4·15	—
				A	3·25	4·08	0·27	0·40	Nil	4·75	0·05
					(2 other exps. gave closely similar results)						
						Cardiac muscle					
27	20	(1·55)	4	B	3·00	3·90	0·30	—	—	4·20	—
				A	2·86	3·80	0·25	0·01	0·02	4·08	0·14
					(1 other exp. gave a closely similar result)						
						Spleen					
29	366	0·80	89	B	3·14	3·47	0·25	—	—	3·72	—
				A	3·06	2·98	0·25	0·36	0·15	3·74	0·08
					(1 other exp. gave a closely similar result)						

B. Before respiration. A. After respiration.

Table 3

Exp.	Tissue		Laevulose mg.	Total sugar (medium) mg.	Total carbohydrate (tissue) mg.	Glucose converted into lactic acid mg.	Carbohydrate oxidized mg.	Total carbohydrate accounted for mg.	Laevulose disappeared mg.	Apparent gain in carbohydrate mg.
					Rat on normal diet					
30	Liver	B	2·39	4·22	1·38	—	—	5·60	—	—
		A	1·41	5·27	0·43	0·40	0·37	6·47	0·98	0·87
	Kidney	B	2·45	3·40	0·42	—	—	3·82	—	—
		A	1·38	2·80	0·42	Nil	0·87	4·09	1·07	0·27
				(2 other exps. gave closely similar results)						
					Rat on high carbohydrate diet					
33	Liver	B	3·03	4·27	1·90	—	—	6·17	—	—
		A	1·44	4·92	1·63	0·38	0·22	7·15	1·59	0·98
	Kidney	B	2·58	3·62	0·45	—	—	4·07	—	—
		A	1·44	2·70	0·45	0·61	0·28	4·04	1·14	−0·03
				(1 other exp. gave a closely similar result)						
					Starved rat					
35	Liver	B	2·31	3·50	0·45	—	—	3·95	—	—
		A	1·17	2·87	0·45	0·41	0·31	4·04	1·14	0·09
	Kidney	B	2·19	3·72	0·50	—	—	4·22	—	—
		A	1·03	3·50	0·40	0·15	0·12	4·17	1·16	−0·05
				(2 other exps. gave closely similar results)						

respiratory exchange was too small for accurate measurement. Had conversion of laevulose into glucose occurred at the same rate, proportional to the O_2 usage, as in liver, it would have been detectable by the direct estimations of laevulose, but nothing can be said as to the production of 'new' carbohydrate in these experiments.

It will be noticed that in all the experiments with liver slices, including those under anaerobic conditions, there was an apparent gain in carbohydrate, a phenomenon which was found with no other tissue except duodenal mucosa. Such an increase in total carbohydrate when liver slices were incubated with Ringer's solution had been previously reported by Gemmill & Holmes [1935], who because of the low R.Q. obtained with liver slices from butter-fed rats, attributed the increase to a conversion of fat into carbohydrate. This was criticized by Cori & Shine [1935] who pointed out that the increase could be attributed to the glycerol and phosphoric ester fraction of the fat. Bach & Holmes [1937] showed that the formation of carbohydrate under their experimental conditions was partially inhibited by insulin and that this fraction appeared to be derived from amino-acids. The precursor of the remaining carbohydrate formed they again suggested was fat.

That the increase in carbohydrate was not due to fortuitous errors of analysis is shown further by Exps. 30–35 (Table 3) which represent simultaneous experiments with liver and kidney slices. In every case liver slices gave an increase in carbohydrate, whilst kidney slices gave an almost perfect balance.

Exps. 30–35 show that the gain in carbohydrate was greatest with the livers from rats which had been on a high carbohydrate diet, and was practically non-existent with the livers from starved rats.

There are several possible precursors of carbohydrate in such a system as that used in liver slice experiments, e.g. protein and protein derivatives, the glycerol fractions of the fats and phospholipins, whilst the formation of reducing trioses from hexoses would produce an apparent gain in carbohydrate.

Since, in liver especially, some of the 'lactic acid' may actually be aceto-acetic and β-hydroxybutyric acids, the figure for glucose converted into lactic acid is probably somewhat high, and so consequently is that for total carbohydrate accounted for at the end of the experiment. Even if the whole of the 'lactic acid'

Table 4

Exp. 38. Estimation of urea before and after the incubation of 100 mg. moist liver slices with 1·5 ml. medium at 37°.

mg. urea N			Glucose equivalent of increase
Initial	Final	Increase	(G/N = 6·25)
0·008	0·012	0·004	0·025
0·004	0·004	Nil	Nil
0·012	0·012	Nil	Nil
0·012	0·028	0·016	0·100

Exp. 39. Estimation of non-protein N. Conditions as in Exp. 38.

mg. non-protein N			Glucose equivalent of increase
Initial	Final	Increase	(G/N = 6·25)
0·140	0·135	Nil	Nil
0·267	0·275	0·008	0·05
0·180	0·200	0·020	0·13

were derived from non-carbohydrate sources, there would still, however, be an apparent gain in carbohydrate.

Only minute increases in urea or non-protein nitrogen could be detected after incubation (Exps. 38 & 39, Table 4), and these could only account for a small fraction of the observed gain in carbohydrate even assuming an optimum conversion of protein into carbohydrate, i.e. a G/N ratio of 6·25.

If hexoses are broken down to reducing trioses, i.e. triosephosphates, or if phosphoric esters are the actual substances which appear as 'carbohydrate' and so account for the apparent increase, there might be some disturbance of the inorganic P/organic P ratio and there should be some increase in the organic acid-soluble phosphate. It would be peculiar if such changes in phosphate were confined to liver tissue when other tissues are also metabolizing carbohydrate. Experiments show very little disturbance of this ratio and very little, if any, increase in organic phosphate.

Table 5

Exp. 40. Acid-soluble P estimations before and after the incubation of 100 mg. moist liver slices with 1·5 ml. medium at 37°. Rat on high carbohydrate diet.

	Initial		Final	
	Inorganic P mg.	Organic P mg.	Inorganic P mg.	Organic P mg.
(*a*)	0·0965	0·0265	0·0937	0·0400
(*b*)	0·094	0·047	0·097	0·044

In one of these experiments there is no evidence of any increase at all in the reducing phosphoric acid esters; in the other, the increase found in the organic acid-soluble phosphate would, if it were all present as newly formed reducing substance (i.e. triosephosphate), account for 0·09 mg. 'glucose' in the calculated balance sheet. If it were triosephosphate formed from a non-reducing precursor (e.g. glycerol or glycerophosphoric acid) this is then the maximum amount of apparent carbohydrate which could be accounted for; if it were formed from a reducing or potentially reducing precursor (glucose or glycogen) only half this would be a gain in 'carbohydrate'. In either case the amount is much less than the apparent gains in carbohydrate actually found. It appears then that only a small part of the 'carbohydrate' which appears during the incubation of liver slices can be derived from protein and amino-acids or by an increase in reducing power due to formation of reducing phosphoric esters. It remains to consider the possibility of carbohydrate formation from fat.

In studying the changes in the fat during incubation, the fat was extracted from the tissue and medium by a mixture of alcohol and ether (3 : 1). Eventually the fatty acids were weighed after a treatment involving saponification, acidification with H_2SO_4 to liberate the fatty acids and their final extraction with light petroleum (B.P. 40–60°). The experiments reported in Table 6, show that in the case of liver slices, an increase in carbohydrate corresponds to a decrease in fatty acids whilst the kidney slices gave neither the apparent gain in carbohydrate nor any decrease in fatty acids. That the increase in carbohydrate cannot be attributed to the glycerol fraction of the fat is clearly shown by these experiments. Assuming the glycerol fraction of a fat to be approximately one-tenth of the molecular weight, then a loss 0·87 mg. of fat (as was found in Exp. 41) would correspond to only 0·09 mg. of carbohydrate derived from glycerol, whereas the actual gain was 0·52 mg.

Table 6

Exp.		evulose mg.	Total sugar (medium) mg.	Total carbohydrate (tissue) mg.	Glucose converted into lactic acid mg.	Carbo-hydrate oxidized mg.	Total carbohydrate accounted for mg.	Laevulose disappeared mg.	Increase in carbohydrate mg.	Total fatty acids mg.	Decrease in fatty acid mg.
				Liver. Medium containing laevulose and glucose							
41	B	3·25	4·12	0·28	—	—	4·40	—	—	5·68	—
	A	1·65	3·76	0·52	0·34	0·33	4·95	1·60	0·55	4·81	0·87
				(1 other exp. gave a closely similar result)							
				Liver. Medium containing glucose only							
45	B	—	4·60	1·22	—	—	5·82	—	—	4·34	—
	A	—	5·65	0·75	0·36	0·10	6·86	—	1·04	3·62	0·72
				(2 other exps. gave closely similar results)							
				Kidney. Medium containing glucose only							
47	B	—	3·00	0·35	—	—	3·35	—	—	3·31	—
	A	—	2·65	0·35	0·09	0·41	3·50	—	0·15	3·40	−0·09
				(1 other exp. gave a closely similar result)							
				Liver. Medium containing glucose only. Atmosphere of N_2							
49	B	—	3·40	0·77	—	—	4·17	—	—	2·75	—
	A	—	3·68	0·45	0·36	—	4·49	—	0·32	1·77	0·98
				(2 other exps. gave closely similar results)							

DISCUSSION

Even if one adds together the amounts of carbohydrate possibly obtainable from protein and amino-acids, increase in phosphoric ester reducing substances, the glycerol of fats and the factor introduced by the formation of acetoacetic and β-hydroxybutyric acids making the total carbohydrate accounted for too high, there is still a very considerable amount of carbohydrate unaccounted for. The quantity involved is such that only the fatty acids would seem capable of being regarded as the source. Incidentally it may be remarked that the increase in carbohydrates with a corresponding decrease in fatty acids is not a property peculiar to experiments in which the medium contains laevulose, but is also found when the medium contains only glucose (Exp. 45).

It is evident that, of the factors considered, only the disappearing fatty acid is quantitatively sufficient to account for the apparent new formation of carbohydrate in liver slices. The further fact that in other tissue slices, in which no carbohydrate formation is detected, there is no loss of fatty acid, considerably strengthens the conclusion that fatty acids are converted into carbohydrate by liver.

There are, however, difficulties in accepting this conclusion without reserve. Thus the observed O_2 consumption is too low to allow the production of the observed carbohydrate from fatty acid together with the oxidation of the remainder of the fat which disappears. It is true that since the formation of carbohydrate and the loss of fatty acids seemed to occur in liver slices anaerobically (Exp. 49, Table 6) one may invoke an unknown hydrogen acceptor and imagine it to be functioning also in the presence of O_2. But this is not entirely satisfactory, and without further proof of the existence of such a hydrogen acceptor there remains a suspicion that some experimental error may have been overlooked.

It is important to remember that the carbohydrate balance sheets were calculated, at a time when the new formation of carbohydrate and the disappearance of fatty acid were unsuspected, on the assumption that the respiratory exchange represented complete oxidation. If fatty acid or anything else is converted into carbohydrate this assumption is unjustified and the calculations are wrong. They cannot be corrected, for, apart from the question of the anaerobic changes which may be occurring, recalculation would involve the assumption of the fatty acid conversion which the figures are designed to prove (or disprove). In any case, such a calculation leads to impossible results, which is not surprising in view of the apparent anaerobic changes in the carbohydrate and fat contents of the system. This error in the calculations does not affect the observation that carbohydrate (or at any rate, reducing substance) is formed in liver slices, for the increase in reducing power (calculated as glucose), plus the lactic acid formed is usually greater than the calculated carbohydrate loss by oxidation. In some experiments the actual reducing power of the system is definitely increased. Anaerobically, there is, of course, no loss of carbohydrate by oxidation, and the increase in reducing power is again real.

The apparent gain in carbohydrate has been observed by so many workers that it may be accepted, but since the gain is one of reducing power only, there is no complete proof that it is really due to formation of hexoses or polysaccharides. The exclusion of phosphoric ester production, does not amount to proof. Similarly the loss of fatty acid seems real and careful checking of the gravimetric analyses by a titrimetric procedure confirms the results and shows the disappearance of fatty acid to be due to complete removal of fatty acid molecules and not to a shortening of the carbon chain. Incidently it is interesting to note that if the whole of the 'glucose converted into lactic acid' could be attributed to the keto-acids, it may account for the loss in fatty acids, since they are in the ratio of about 2·5 to 1, as are their molecular weights. The unexpected suggestion of an anaerobic conversion of fatty acid into carbohydrate with its corollary of a non-oxygen hydrogen acceptor is, however, disturbing, and indicates that much more work (which war conditions prevent) is required.

SUMMARY

1. It is shown that the liver and kidney, but not the brain, spleen, cardiac muscle, skeletal muscle or duodenum, are capable of converting laevulose into glucose or glycogen. The conversion occurs only in the presence of oxygen and of intact liver cells.

2. All experiments with liver and duodenal mucosa showed an apparent gain in carbohydrate, whilst the other tissues did not.

3. The increase in carbohydrate was greatest with the tissues of rats which had received a high carbohydrate diet and least with those from starved rats.

4. The increase in carbohydrate corresponded to a decrease in fatty acid, but though the evidence is suggestive it is not regarded as proved that the fatty acid is converted into carbohydrate.

5. It is suggested that some hydrogen acceptor other than oxygen may, in these experiments have been concerned in a disappearance of fatty acids and production of carbohydrate.

REFERENCES

Ashford (1933). *Biochem. J.* **27**, 903.
Bach & Holmes (1937). *Biochem. J.* **31**, 89.
Bodansky (1923). *J biol. Chem.* **56**, 387; **58**, 515, 799.
Bollman & Mann (1931). *Amer. J. Physiol* **96**, 683.
Bornstein & Volker (1929). *Biochem. Z* **209**, 103.
Burget, Moore & Lloyd (1932). *Amer. J. Physiol.* **101**, 16, 565.
Cori & Shine (1935). *Science*, **82**, 134.
— (1936). *J. biol. Chem.* **104**, 21.
Corley (1929). *J. biol. Chem.* **81**, 81.
Dickens & Šimer (1931). *Biochem. J.* **25**, 973.
Gemmill & Holmes (1935). *Biochem. J* **29**, 338.
Goda (1937). *Biochem Z* **294**, 259.
—— (1938). *Biochem. Z.* **297**, 134.
Griesbach (1929). *Z. ges. exp. Med.* **65**, 172.
Griffith & Waters (1936). *Amer. J. Physiol.* **117**, 134.
Herbert (1938). *Biochem. J.* **32**, 815.
Herring, Irvine & Macleod (1924). *Biochem. J.* **18**, 1023.
Isaac (1920). *Med Klinik.* **14**, 1207.
Krebs & Henseleit (1932) *Hoppe-Seyl. Z.* **210**, 33.
Lampson & Wing (1926). *J. Pharmacol.* **28**, 399.
Maclean & Smedley (1912). *J. Physiol.* **45**, 462.
Mann & Magath (1922). *Arch. Intern. Med.* **30**, 73.
McGuiran (1908). *Amer. J. Physiol.* **21**, 334.
Noble & Macleod (1924). *J. Physiol.* **64**, 547.
Ochoa (1930). *Biochem. Z.* **227**, 116.
Okamura (1938). *Japan J. Med. Sci.* ii, **4**, 101.
Oppel (1929). *Biochem. Z.* **205**, 47.
Reilly, Nolan & Rusk (1898). *Amer. J. Physiol.* **1**, 395.
Steinberg (1927). *Pflug. Arch. ges. Physiol.* **217**, 686.
Stewart & Gaddie (1934). *J. Physiol.* **80**, 457.
Verzár & McDougal (1936). Absorption from the intestine. London: Longmans Green & Co.

26. OBSERVATIONS ON THE SERUM PHOSPHATASE OF CATTLE AND SHEEP

BY WILLIAM MILLER ALLCROFT AND
SYDNEY JOHN FOLLEY

From the Ministry of Agriculture Veterinary Laboratory, Weybridge, and the National Institute for Research in Dairying, University of Reading

(*Received 28 January 1941*)

THE presence in blood plasma or serum of an enzyme capable of hydrolysing esters of phosphoric acid optimally at an alkaline reaction, has been known for many years [see McCollum & Hart, 1908], and it is now evident that this phosphatase is the phosphomonoesterase A_1 in the classification of Folley & Kay [1936, 1]. Thus, for example, Gutman & Gutman [1940] state that normal human blood serum contains very little 'acid' phosphatase. The amount of phosphatase present in blood is considerably less than in such tissues as intestinal mucosa, bone epiphysis, kidney and mammary gland [see Folley & Kay, 1936, 1]. Normal values of serum phosphatase in arbitrary units have been given for man and certain animals by Kay [1930, 1] and for man and dog by King & Armstrong [1934], but as yet only limited data have been presented for the activity of this enzyme in the blood of farm animals.

Serum phosphatase is raised above normal in certain generalized bone disorders in man, such as rickets (infantile, renal and adult), generalized osteitis fibrosa, osteitis deformans and obstructive jaundice [see Folley & Kay, 1936, 1], and it has also been claimed that it is raised in certain other diseases such as tuberculosis and carcinoma, but the evidence here is less conclusive. Since serum phosphatase values have proved of diagnostic value in disordered Ca and P metabolism in man it was hoped that the same might hold in farm animals, as was suggested, on the basis of a limited number of observations on sheep, by Auchinachie & Emslie [1933].

Before the possible diagnostic value of phosphatase determinations in farm animals could be assessed it was obviously essential to know the limits of probable variation in the normal animal. This paper records data obtained for normal cattle and sheep at the National Institute for Research in Dairying, Shinfield, and the Veterinary Laboratory, Weybridge, these institutions maintaining close co-operation throughout the work.

The data for cattle were in the first instance obtained at Shinfield during the years 1933–5 in the course of investigations into the possible relationship between blood and milk phosphatases [see Folley & Kay, 1936, 2] and were later supplemented by data obtained on other herds to see if certain peculiarities characteristic of the Shinfield cows were also shown under different conditions of herd management and by other breeds, and by some data from Weybridge.

During the period 1934–5 investigations which included serum phosphatase determinations were made at Weybridge into 'cripples' of lambs, a disease described by Lyle Stewart [1933] upon which extensive biochemical studies were made by Piercy [1934, 1, 2]. It was hoped that serum phosphatase estimations would aid diagnosis of the bone disorder for which there is evidence in this disease, but the data showed wide variations uncorrelated with the incidence of 'cripples'. A large-scale experiment on the aetiology of 'pining' in Northumberland sheep which was jointly conducted by Mr W. Lyle Stewart and Dr A. P. Ponsford of Newcastle-upon-Tyne and Dr H. H. Green and Dr E. L. Taylor of Weybridge gave an opportunity of acquiring an additional large number of serum samples for phosphatase determinations. 'Pining' is a loose term for a wasting disease of sheep associated in Northumberland with helminth infection and mineral deficiency, possibly of cobalt. In all 240 ewes and lambs were bled, some of them several times, and over 900 samples were available for blood studies in various directions.

It should be stated here that these sheep were apparently normal and showed no evidence of 'pining' over the period in which the phosphatase data were accumulated. These data were supplemented by a further 220 samples from normal ewes in other flocks.

EXPERIMENTAL

All blood samples were obtained from the jugular vein, with the aid of partial stasis caused by a choke-rope in the case of cattle and by thumb pressure in the case of sheep.

Serum phosphatase was determined in both laboratories by the method of King & Armstrong [1934] which involves incubation for 30 min. at 37°. Two slight modifications were made: (*a*) sufficient phenol was added to the phenol reagent used for blank determinations to give a colour readily readable in the ordinary colorimeter, and (*b*) before making the colorimetric reading all tubes were immersed in the water bath at 37° for 45 min. to develop the blue colour.

Blood samples taken near either laboratory were left until the next day to ensure complete coagulation and the sera were then analysed. Samples taken at distant centres were sent by rail in special ice-boxes. When a batch of samples was too large to be dealt with in one day, the sera not analysed on the first day were kept for one or, on some occasions, two days in the refrigerator. It was previously found by one of us (W. M. A.) that storage of serum in the refrigerator at 2° for periods up to 4 days made no appreciable difference to its serum phosphatase activity. All haemolysed sera were discarded as unfit for phosphatase determination.

RESULTS

The joint data are considered from a number of aspects, and for simplicity and economy of space the main features are presented in the form of distribution tables. In compiling these tables the values have been classified into groups covering 5 units for values from 0 to 30 units, 10 units for values between 30 and 50 units and thereafter in larger groups according to the data available. While all values were originally recorded to the first place of decimals, in the distribution tables they are, for simplicity, given to the nearest integer. Throughout this paper the term phosphatase refers to the phosphomonoesterase A_1 [Folley & Kay, 1936, 1] and all values are recorded in King & Armstrong units per 100 ml.

It will be noted that the number of samples exceeds the number of animals in the case of both cattle and sheep. This is due to the fact that 22 of the Dairy Shorthorn cows from one herd and 48 of the sheep from the 'pining' experiment at Newcastle-upon-Tyne were bled on several occasions. All the values so obtained have been included in the analysis of variability of results, since we consider that this affords a better criterion of the extreme range of variation to be met with in normal animals. The alternative of entering the mean of the several values for each such animal in the distribution tables was considered and rejected, since this would tend to mask the extreme limits of variation. Only for investigation of the possible association between milk yield and serum phosphatase has the average value for each cow been taken.

Range of serum phosphatase in the apparently normal cow and ewe

For this study there were available 367 samples from 294 cows in 8 different herds maintained under varying systems of first-class dairy management, and 119 samples from the same number of Hereford cows kept under range conditions all the year round with the bare minimum of supplementary feeding in winter. For the ewe there are 555 samples from a number of pure-bred and cross-bred flocks. The overall ranges of values were 0·3–114·3 units for cows and 3·0–166·1 units for ewes. The summarized distribution data are given in Table 1.

Table 1. *Distribution of serum phosphatase values, actual and on percentage basis, in normal cows and ewes*

Serum phosphatase units	Dairy breeds		Herefords		All cows		All ewes	
	No.	%	No.	%	No.	%	No.	%
0·0– 4·9	59	16·1	38	31·9	97	20·4	11	2·0
5·0– 9·9	117	31·9	60	50·4	177	36·4	139	25·1
10·0–14·9	60	16·3	12	10·1	72	14·8	146	26·2
15·0–19·9	27	7·4	1	0·8	28	5·8	66	11·9
20·0–24·9	22	6·0	3	2·5	25	5·1	70	12·6
25·0–29·9	17	4·6	3	2·5	20	4·1	37	6·7
30·0–39·9	20	5·4	2	1·7	22	4·5	40	7·2
40·0–49·9	20	5·4	—	—	20	4·1	28	5·0
50·0–99·9	22	6·0	—	—	22	4·5	12	2·2
100·0 and over	3	0·8	—	—	3	0·6	6	1·1
Total no.	367		119		486		555	
Below 15 units		64·3		92·4		71·2		53·3
Below 40 units		87·7		100·0		90·7		91·7

The first thing to be noted in Table 1 is that, despite the wide overall range in each species, the greater proportion of values for both cows and ewes are under 40 units, though a small proportion of apparently normal animals show values above this limit, even exceeding 100 units in about 1 % of cases. Among the cows it will be seen that all values for the Hereford breed are below 40 units whereas there are 12·3 % of values over 40 units among the dairy breeds. Further discussion of this fact is left until later when possible breed differences will be considered.

Whereas in the data for cows 71·2 % of values are below 15 units and 19·5 % between 15 and 40 units, the corresponding figures for ewes are 53·3 and 38·4 % respectively. Considering now the values under 10 units, these are 56·8 % for cows and 27·1 % for ewes. These figures show that the scatter of values below 40 units is greater in ewes than in cows and suggests a species difference with the median value for ewes higher than that for cows.

The wide variations in apparently normal ewes and cows, particularly of the Dairy Shorthorn breed, revealed by the data in Table 1 make it evident that the diagnostic value of determinations of serum phosphatase in cases of suspected disorders of Ca and P metabolism in farm ruminants is likely to be extremely limited.

It is interesting to compare the normal ranges found by us in cows and ewes with the figures given by King & Armstrong [1934] for man and the dog. This is possible since the units were the same in both investigations. The normal range found by King & Armstrong for 24 normal human subjects between 19 and 42 years of age was 3·7–13·1 units, the majority falling between 5 and 10 units. For 21 dogs they found a wider range, two of them showing values of 27·4 and 32·1 units respectively, findings reminiscent of our results in ruminants.

Relation between serum phosphatase and age

Kay [1930, 1], Smith & Maizels [1932] and Stearns & Warweg [1933] reported that serum phosphatase was higher in children than in adults and similar age differences have been found in the rabbit and rat [Kay, 1930, 1] and dog [Bodansky, 1934]. The higher level in infancy may be associated with the more rapid skeletal development at this stage of life. Mulay & Hurwitz [1938] studied the plasma phosphatase in 300 human subjects ranging in age from the time of birth to 86 years and found comparatively high values between 1 and 15 years, a decrease between 15 and 25 years and thereafter a fairly constant level. Our data in general are in accord with the findings of these workers.

The data for Dairy Shorthorn cows were classified among the age groups, 2–4 years, 4–6 years and over 6 years. To these three groups were added a small group of heifers 9–24 months old, and three sets of results obtained on the same population of calves at three age periods. These data are given in Table 2.

Table 2. *Distribution of serum phosphatase in different age groups of Dairy Shorthorn cattle*

Serum phosphatase units	Calves 8 weeks	Calves 17 weeks	Calves 27 weeks	Heifers 9–24 months	Cows 2–4 years	Cows 4–6 years	Cows over 6 years	Total of calves	Total of heifers	Total of cows	Total of cows and heifers
0·0– 4·9	—	—	—	—	1	3	1	—	—	5	5
5·0– 9·9	—	—	1	—	15	12	6	1	—	33	33
10·0–14·9	2	3	14	8	8	3	3	19	8	14	22
15·0–19·9	8	14	8	4	2	3	3	30	4	8	12
20·0–24·9	6	8	1	2	1	4	2	15	2	7	9
25·0–29·9	8	2	—	3	—	—	2	10	3	2	5
30·0–39·9	3	3	—	—	—	1	1	6	—	2	2
40·0–49·9	4	—	—	1	2	—	1	4	1	3	4
50·0–99·9	1	—	—	—	1	—	6	1	—	7	7
100·0 and over	—	—	—	—	1	—	—	—	—	1	1
Total no.	32	30	24	18	31	26	25	86	18	82	100

Among the calves, data for which are recorded in the first three columns, there is at first a wide scatter of values from 10 to 50 units with a subsequent tendency towards a closer scatter and a downward trend of values. The individual records of 24 of the calves which were bled on all three occasions show that 22 of them yielded diminishing values at successive samplings, while the values for the other 2 were singularly steady throughout the period. The heifers form a small intermediate group and the three age groups of cows show a preponderance of even lower values up to 6 years old, after which there is again a number of higher values. The groups of animals studied here are too small to warrant much discussion, but the data do suggest that in cattle, after maturity, there is not likely to be any regular trend in the serum phosphatase level. It is admitted that the composite picture of the relation between age and serum phosphatase shown in Table 2 is far from complete, and the data do not permit the drawing of hard and fast conclusions. The only way to obtain a satisfactory picture of the change of serum phosphatase with advancing age is to study a large number of animals from birth to old age, a long and difficult task in the case of cattle.

In addition to the data on cattle there are similar data for sheep and these are summarized in Tables 3, 4 and 5.

Table 3. *Serum phosphatase data in four Suffolk × Dorset Horn lambs from birth to about 10 weeks old*

	Age in days							
Lamb no.	1	7	14	21	27	36	51	67
911	264	90	57	45	72	73	68	32
912	181	135	47	65	89	86	72	30
913	226	64	64	40	43	22	18	13
914	229	134	127	84	54	41	31	16
Average	225	106	74	58	65	55	48	23

Table 4. *Average values of serum phosphatase in 10 Cheviot lambs from 25 to 185 days old*

Average age in days	25	43	51	58	71	85	112	161	185
Range of phosphatase	64–165	60–127	41–150	41–115	32–77	27–73	11–27	9–34	5–33
Average phosphatase	105	84	92	83	50	40	20	20	13

Table 5. *Percentage distribution of serum phosphatase in 7 age groups of sheep from Newcastle 'pining' experiment*

Serum phosphatase units	5 months old 139 samples	9 months old 43 samples	13 months old 69 samples	17 months old 89 samples	3 years old 92 samples	3¾ years old 87 samples	4 years old 93 samples
0·0– 4·9	—	—	—	—	1·0	—	2·1
5·0– 9·9	4·3	2·3	1·4	16·9	21·7	6·9	42·0
10·0– 14·9	8·6	23·2	23·2	24·7	23·9	41·4	26·9
15·0– 19·9	15·1	23·2	31·9	16·9	20·7	18·4	5·4
20·0– 24·9	18·7	16·3	8·7	15·7	6·5	15·0	8·6
25·0– 29·9	15·1	23·2	10·1	7·9	7·6	5·7	3·2
30·0– 39·9	21·6	7·0	18·9	11·2	7·6	5·7	6·4
40·0– 49·9	12·9	2·3	1·4	1·1	6·5	4·6	2·1
50·0– 99·9	1·4	2·3	2·9	3·4	3·3	1·1	2·1
100·0–199·9	2·2	—	1·4	—	1·0	1·1	1·1
200·0–349·9	—	—	—	1·1	—	—	—
Under 15 units	12·9	25·2	25·6	41·6	46·6	48·3	71·0
Under 40 units	83·4	95·4	94·3	94·4	89·2	93·2	94·7

Tables 3 and 4 show that at birth the lamb has a very high serum phosphatase which falls very rapidly in some cases and more slowly in others, the fall continuing up to about 6 months of age. There is a decided difference in actual level in the two groups at corresponding ages but the trend is much the same in each group. These groups are too small to enable us to say whether or not this difference is likely to be connected with the breed.

It will be noted (see Table 5) that while the percentage of values under 40 units is fairly constant after 9 months of age in the sheep, there is a more or less steady increase in the number of values under 15 units, which rises from 12·9% at 5 months to 71·0% at 4 years. This shows a progressive condensing of the scatter of phosphatase values with advancing age, a finding not observed in the smaller and differing cattle populations studied. The general picture emerging from the data on sheep resembles in part the finding in cattle but is more clear cut, possibly because the life of the sheep is shorter than that of the cow and a larger proportion of the life-span has been studied. The sheep data can furthermore be considered as of more significance than the data on cows in that the same population of sheep was studied over the last three ranges of age, whereas the data in Table 2 were obtained on three different and smaller groups of cattle. The possible existence of a species difference in the relation between age and serum phosphatase, nevertheless, cannot be entirely ignored.

The serum phosphatase level of the individual

In view of the wide overall range of values found among cattle and sheep it was considered important to know also what variability occurred from time to time in the same individual. A certain number of cows, ewes and lambs have been bled on a number of occasions and the full data available for these animals are presented in Tables 6 and 7.

Table 6. *Serum phosphatase values of 11 dairy Shorthorn cows at successive samplings*

Name of cow	1934			1935						Range of values	Average
	1. xii.	5. xii.	10. xii.	14. i.	23. i.	11. ii.	11. iii.	8. v.	18. xi.		
Campion 2	23	—	24	21	—	15	17	24	35	15–35	23
Carlet 3	—	—	58	—	—	57	56	53	114	53–114	68
Lily 3	—	11	11	10	—	11	17	11	12	10–17	12
Flora 1	—	—	9	8	—	9	9	—	—	8–9	9
Linda 5	—	—	61	—	—	47	58	68	—	47–68	58
Patricia	8	—	11	—	—	10	10	13	—	8–13	10
Peach 3	48	—	44	48	—	43	53	70	63	43–70	53
Peach 5	—	17	17	—	29	28	27	21	44	17–44	26
Pippin	—	—	22	26	23	18	27	27	—	18–27	24
Red Rose	—	—	9	—	—	8	9	12	12	8–12	10
Rosalie	—		42	44	—	40	49	35	67	35–67	46

From the data in these tables it appears that the general level of serum phosphatase in the normal adult individual remains sensibly constant over periods of the order of at least 1 year in the case of cows and 18 months in the case of ewes and lambs. An animal showing a low serum phosphatase activity at the first sampling has usually continued to do so with moderate fluctuations around the mean level, whereas an animal showing a high initial value always showed a high value. It is further clear that those animals showing high values, e.g. Carlet 3, Linda 5, Peach 3 and Rosalie in Table 6 and Lambs 46, 71, 75, and 136 and Ewe 26 in Table 7, also showed considerably wider fluctuations in serum phosphatase than animals showing a generally low level. As far as we are able to tell, the level of serum phosphatase of the individual, whether high or low, does not seem to be associated with any clinical abnormality. The wide variation in general level of serum phosphatase among apparently normal cattle and sheep is, however, a matter of great interest, and the possibility that changes in serum phosphatase might be associated with physiological conditions such as pregnancy and lactation was examined.

Table 7. *Serum phosphatase values in 41 sheep at 7 different samplings showing individual variation*

No.	Nov. 1936	Dec. 1936	Jan. 1937	Feb. 1937	July 1937	Nov. 1937	Mar. 1938	Range	Average
				1936 lambs					
2	7	14	9	11	12	13	10	7– 14	11
6	13	19	13	15	19	18	8	8– 19	15
12	40	35	30	23	30	33	17	17– 40	30
15	20	36	25	33	31	30	26	20– 36	29
39	20	16	16	12	16	12	11	11– 20	15
40	10	15	12	13	18	10	11	10– 18	13
44	8	17	10	10	15	7	8	7– 17	11
46	102	129	205	156	52	131	89	52–205	124
67	27	22	8	9	23	15	8	8– 27	16
71	46	120	38	55	38	72	44	38–120	59
75	122	146	57	119	—	19	—	19–146	93
77	13	17	13	9	15	11	9	9– 17	12
99	47	38	20	25	29	28	23	23– 47	30
101	26	19	14	13	29	14	11	11– 29	18
102	27	21	13	17	12	15	9	9– 27	16
108	35	30	14	16	21	17	8	8– 35	20
131	39	26	21	23	26	25	15	15– 39	25
134	50	19	13	9	19	16	11	9– 50	19
136	142	—	—	—	172	316	—	142–316	—
139	36	48	24	35	14	30	37	14– 48	32
142	68	46	15	28	—	40	25	15– 68	36
				1936 ewes					
19	17	18	14	10	14	9	8	8– 18	13
22	7	10	8	8	9	9	9	7– 10	8
26	110	166	62	135	51	113	114	51–166	107
32	41	49	31	25	45	29	37	25– 49	34
49	9	11	7	8	11	6	6	6– 11	8
52	29	15	15	14	37	21	16	14– 37	21
60	20	14	9	7	10	9	8	7– 20	11
62	52	34	21	22	43	25	15	15– 52	30
84	11	12	5	9	10	10	6	5– 12	9
86	17	9	14	15	—	3	9	3– 17	11
91	26	27	13	16	19	16	9	9– 27	18
96	42	25	19	17	25	22	21	17– 42	24
113	17	22	7	7	10	14	8	7– 22	12
120	44	26	23	28	36	33	22	22– 44	30
122	16	17	8	13	14	11	7	7– 17	12
124	43	17	13	24	23	22	15	13– 43	23
149	9	12	6	8	11	11	7	6– 12	9
152	9	8	5	5	9	6	6	5– 9	7
154	15	18	5	7	17	12	11	5– 18	12
156	17	20	10	7	19	7	9	7– 20	13

Serum phosphatase and pregnancy in the dairy cow

Wilson & Hart [1932] and Auchinachie & Emslie [1933] claim that serum phosphatase is lower in pregnant than in non-pregnant cows, while the latter authors also found the same to hold for ewes. Vermehren [1939] stated that in women serum phosphatase is increased in the latter half of pregnancy, reaching a maximum just before parturition and falling sharply immediately *post partum*. He further states that the level during lactation is somewhat higher than the normal level.

Our available data for the dairy cow have been classified in Table 8 in two ways: (*a*) according as to whether or not the cow was pregnant when sampled, and (*b*) the values for pregnant cows according to stage of pregnancy.

Table 8. *Serum phosphatase of dairy cows in relation to pregnancy*

Serum phosphatase units	Pregnant		Non-pregnant		Nos. at various stages of pregnancy		
	No.	%	No.	%	0–95 days	96–190 days	191–285 days
0·0– 4·9	3	5·2	1	1·6	1	1	1
5·0– 9·9	10	17·5	27	43·5	2	6	2
10·0–14·9	15	26·3	11	17·7	9	5	2
15·0–19·9	5	8·8	8	12·9	2	1	2
20·0–24·9	3	5·2	4	6·4	2	1	—
25·0–29·9	4	7·0	3	4·8	2	1	1
30·0–39·9	1	1·8	1	1·6	1	—	—
40·0–49·9	9	15·8	1	1·6	1	6	2
50·0–99·9	6	10·5	5	8·0	3	1	1
100·0 and over	1	1·8	1	1·6	—	—	1
Totals	57		62		23	22	12
Under 15 units		49·0		62·8	12	12	5
Under 40 units		71·9		88·8	19	15	9

Comparing the serum phosphatase levels in pregnant and non-pregnant cows in Table 8, it is noted that there is a higher proportion of low values among the non-pregnant cows and that the scatter of values is wider among the pregnant cows, suggesting at first sight a tendency towards higher serum phosphatase values in pregnancy. Our data, therefore, are more in line with those of Vermehren [1939] on women than with those of Wilson & Hart [1932] and Auchinachie & Emslie [1933] on cattle and sheep. It must, however, be admitted that our data are not sufficiently extensive to preclude the possibility of a decrease in serum phosphatase in late pregnancy such as was suggested by the results of Auchinachie & Emslie [1933] on six cows sampled during the fifth and eighth months of pregnancy. The data given in the last three columns of Table 8 do not suggest the existence of any pronounced relation between stage of pregnancy and serum phosphatase level. This, taken in conjunction with the individual records of cows sampled when pregnant and non-pregnant, suggests that the preponderance of low values in non-pregnant cows may be due to chance, though the possibility remains that the physiological condition of pregnancy irrespective of the stage reached is associated with a tendency towards an increase in serum phosphatase.

Serum phosphatase of the dairy cow in relation to milking capacity

In searching for possible causes of the wide individual differences in serum phosphatase of dairy cows it was thought that strain on the Ca and P metabolism due to the large daily excretion of Ca in the milk of high-yielding cows might be reflected in an abnormally high serum phosphatase level [cf. Vermehren, 1939]. Accordingly, available data relating to milk yield and serum phosphatase are presented in Tables 9 and 10.

In Table 9 the cows are classified into two groups, according to whether the yield in the lactation previous to sampling was more or less than 7500 lb.

Though the average lactation yields of the two groups of cows differ widely, the distributions of serum phosphatase values are so similar as to suggest that there is no association between milk yield and serum phosphatase. This conclusion is confirmed by the more detailed data (see Table 10) on 11 Dairy Shorthorn cows of which 5 gave over 9000 lb. and 6 gave under 9000 lb. in the previous lactation.

Table 9. *Average values of serum phosphatase in dairy cows of two different grades of milking capacity*

Serum phosphatase units	Total milk yield in lactation previous to the samplings: Under 7500 lb. No.	%	Over 7500 lb. No.	%
0·0– 4·9	24	29·3	16	26·3
5·0– 9·9	29	35·4	21	34·4
10·0–14·9	9	11·1	8	13·2
15·0–19·9	6	7·3	4	6·5
20·0–24·9	5	6·1	3	4·9
25·0–29·9	1	1·2	2	3·3
30·0–39·9	2	2·4	1	1·6
40·0–49·9	3	3·6	1	1·6
50·0–99·9	3	3·6	5	8·2
Totals	82		61	
Highest yield in lb.	7463		16454	
Lowest yield in lb.	1142		7626	
Average yield in lb.	5830		9224	

Table 10. *Serum phosphatase of 11 Dairy Shorthorn cows of different grades of milking capacity*

Cows giving over 9000 lb. in last lactation: Name	Milk yield lb.	Range of serum phosphatase	Average serum phosphatase	Cows giving under 9000 lb. in last lactation: Name	Milk yield lb.	Range of serum phosphatase	Average serum phosphatase
Carlet 3	13036	53–114	68	Pippin	8864	18–27	24
Peach 3	12358	43– 70	53	Red Rose	8638	8–12	10
Lily 3	9759	10– 17	12	Linda 5	8438	47–68	58
Peach 5	9593	17– 44	26	Patricia	8000	8–13	10
Rosalie	9321	35– 67	44	Campion 2	6868	15–35	23
				Flora 1	4043	8– 9	9
Average	10813		40·6		7142		22·3

Table 11. *Serum phosphatase data of 9 herds representing 6 breeds of cattle*

Serum phosphatase units	Dairy Shorthorn herds				Guernsey herds			Other dairy breed herds				Herefords
	S_1	S_2	S_3	Total	G_1	G_2	Total	D	A	F	Total	H
0·0– 4·9	—	2	8	10	4	3	7	—	16	26	42	38
5·0– 9·9	20	17	18	55	18	27	45	9	4	4	17	60
10·0–14·9	22	2	9	33	8	14	22	3	—	2	5	12
15·0–19·9	11	2	5	18	2	6	8	1	—	—	1	1
20·0–24·9	10	—	5	15	—	7	7	—	—		—	3
25·0–29·9	9	—	6	15	—	1	1	1	—		1	3
30·0–39·9	3	2	10	15	—	3	3	2	—	-	2	2
40·0–49·9	10	2	4	16	—	3	3	1	—	—	1	—
50·0–99·9	12	4	5	21	—	1	1	—	—	—	—	—
100·0 and over	2	—	1	3	-		—	—	—	—	—	-
Totals	99	31	71	201	32	65	97	17	20	32	69	119

Normal range of serum phosphatase in various breeds of cattle

Data are available (see Table 11) for cows of 6 breeds comprising 3 herds of Dairy Shorthorns (S_1, S_2 and S_3), 2 herds of Guernseys (G_1 and G_2), and 1 herd each of South Devons (D), Ayrshires (A), British Friesians (F) and Herefords (H).

From these data it appears that the serum phosphatase distribution for Dairy Shorthorns differs from that for cows of other breeds in that it is the only breed exhibiting values over 100 units. Further, about 10 % of values for Shorthorns lie between 50 and 100 units compared with only 1 % in the case of Guernsey cows and none in the other breeds. This wide scatter of values in the Dairy Shorthorn was first observed in herd S_1, and herds S_2 and S_3 were then studied to find out if the peculiarity was a general characteristic of the breed or merely a peculiarity of the particular herd associated with conditions of management or breeding. Since, however, the distribution was similar in all 3 herds it seems fair to assume that it is a genuine breed characteristic.

Data for Ayrshire and British Friesian cattle are comparatively slender, but it is noteworthy that the values for these breeds are all under 15 units with the great majority of values under 5 units. Intermediate between these breeds and the Dairy Shorthorns come the Guernseys, South Devons and Herefords, the last-mentioned being a 'beef' breed. The herd studied by us was at grass all the year round and kept for calf rearing only. We do not propose to comment further on this breed difference since at present no explanation for it can be offered.

DISCUSSION

One feature of the data which have been summarized in the foregoing tables is of such interest as to merit further discussion. We refer to the surprisingly scattered distribution of the serum phosphatase values in ewes and in cows of the Dairy Shorthorn, and, to a less extent, the Guernsey and South Devon breeds. It is difficult to give any adequate explanation of this phenomenon though one or two possibilities will be considered.

There is a certain amount of evidence which suggests that individual differences in the level of thyroid activity might be responsible for the wide variation in serum phosphatase levels of apparently normal individuals. Kay [1930, 2] found that the serum phosphatase was raised above normal in cases of exophthalmic goitre in man. Scoz & Marangoni [1934] observed a transient rise in plasma phosphatase in one dog after thyroxine treatment. Folley & White [1936] noted an increase in serum phosphatase in lactating cows during thyroxine treatment, but concluded that the changes were not large enough to account for the individual variations in serum phosphatase which had been observed at Shinfield among Dairy Shorthorn cows.

A second possibility is opened up by the observation of Folley [1936] that injection of large doses of oestrogenic hormones caused a temporary rise of the order of 50 % in serum phosphatase accompanied by a fall in serum Ca in lactating cows. These changes were interpreted as indicating that oestrogens are capable of exerting an influence on the skeletal system, a contention which has since been repeatedly confirmed [cf. Gardner & Pfeiffer, 1938]. Despite the fact that the doses of oestrogen used in Folley's [1936] experiments were unphysiological and the changes in serum phosphatase not large, the remote possibility remains that individual differences in serum phosphatase might be due in some cases to differences in ovarian activity.

Lastly we would discuss the possibility that the high phosphatase activity of some sera is due to the presence of a phosphatase activator. This possibility was considered by one of us (S. J. F.) but discarded on the basis of evidence acquired in 1935. It was claimed by Thannhauser *et al.* [1937, 1] that ascorbic acid was such an activator, but King & Delory [1938] failed to confirm this and the claim was withdrawn [Thannhauser *et al.* 1938, 1]. In view of the more recent paper of Thannhauser *et al.* [1938, 2] in which the authors claim to have obtained evidence of the presence in sera showing high phosphatase activity of a substance which, when added to normal sera, causes an increased hydrolysis of phosphoric ester, further discussion of this question seems called for.

In investigating this question at Shinfield in 1935, a serum of relatively high phosphatase activity and two of normal or low activity were used. King & Armstrong [1934] have shown that phosphatase estimations carried out by their method on undiluted sera showing an activity of 60 or more of their units per 100 ml., tend to be low owing to inhibition of the activity of the enzyme by the inorganic phosphate produced by the hydrolysis. Accordingly the high phosphatase serum was diluted with an equal volume of normal saline, this being the normal procedure specified by King & Armstrong for sera showing high phosphatase activity.

The phosphatase activities of the three sera, as well as of mixtures of equal parts of diluted high phosphatase serum with each of the other two, were determined. It was considered that if the highly active serum contained an activator, the activity of a mixture of this with a serum of normal activity would be greater than the arithmetic mean of the activities of the two components. This was essentially the procedure later adopted by Thannhauser *et al.* [1938, 2] to investigate this question. The actual figures obtained are given in Table 12, from which it will be seen that the differences between the theoretical and actual values for the phosphatase activities of the mixtures are well within the limits of experimental error. It would therefore appear that the relatively high phosphatase activity of one of these three sera cannot be ascribed to the presence of an enzyme activator.

Table 12. *Effect of mixing sera of different phosphatase values*

Actual values: serum 1 (diluted 1 : 1), 24·7 units per 100 ml.; serum 2, 9 7 units per 100 ml.; serum 3, 8·9 units per 100 ml.

	Theoretical value	Value found
By mixing equal volumes of 1 and 2	17·2	17·7
By mixing equal volumes of 1 and 3	16·9	17·3
By mixing equal volumes of 2 and 3	9·3	9·2

Thannhauser *et al.* [1938, 2] used a method for the determination of phosphatase activity [see Thannhauser *et al.* 1937, 1] based on the method of Bodansky [1933] but the details of technique are not given fully. In their paper they report that the amount of phosphoric ester hydrolysed by mixtures of equal volumes of normal sera agreed closely with expectation but that when mixtures of normal sera and highly active sera were used the phosphate produced exceeded the expected amount. This was taken as evidence of the presence of a phosphatase activator in the pathological sera. In the absence of full experimental details it is hard to make critical comment on their data, but the fact that the more the highly active serum was diluted with normal serum the more the phospholytic activity of the mixture exceeded the theoretical value, suggests that the true activities of the highly active sera might have been underestimated, on account of the slowing up of the rate of hydrolysis due to the inhibiting action of the end-products, a point guarded against in the technique used by us. Against this possible explanation must be set the statement that when four times as much serum was used, the quantity of ester hydrolysed was proportionately greater, which, if other conditions such as concentration of substrate, total volume of liquid etc. were the same in both cases, would preclude the likelihood of their original determinations on highly active sera having been in error due to the inhibitory action on the enzyme of the inorganic phosphate liberated. A further point should be borne in mind. The highly active sera of Thannhauser and his colleagues were obtained from cases of Paget's disease in man and from dogs which had undergone ligation of the common bile duct, whereas the results in Table 12 were obtained with sera from clinically normal cows. It is possible that in Paget's disease, or after ligation of the common bile duct, the blood may contain a phosphatase activator which is absent from blood showing high phosphatase activity due to other causes, or that such an activator may be present in highly active sera from man and carnivores but not from ruminants. In support of this possibility it may be mentioned that there is some evidence of species differences as regards changes in serum phosphatase. Thus Thannhauser *et al.* [1937, 2] claim that ligation of the common bile duct causes an increase in serum phosphatase in dogs but not in cats, while rickets is accompanied by an increase in serum phosphatase in man but not in the rat (Prof. H. D. Kay, personal communication). Whatever may be the true explanation of the results of Thannhauser and his colleagues, the postulation of the presence of an activator in highly active sera does not seem to be a likely explanation of the wide range of values encountered among apparently normal cows of the Dairy Shorthorn breed and among ewes.

SUMMARY

1. In apparently normal cows and ewes the variations in serum phosphatase, 0·3–114·3 and 3·0–166·1 units per 100 ml. respectively, are so wide as to lead to the conclusion that its determination can be of little diagnostic significance.

2. Cows of the Dairy Shorthorn breed show a wider range of values and a greater proportion of high values than cows of four other dairy breeds and one beef breed. No explanation of this breed difference can be offered.

3. Though the serum phosphatase may differ widely from animal to animal the general level of the individual remains sensibly constant over long periods of time.

4. In both cattle and sheep serum phosphatase activity progressively decreases with advancing age until maturity is reached. Thereafter cattle show little alteration but there is some evidence in sheep of a further slight decrease with advancing age.

5. There is some evidence that pregnancy *per se* is accompanied by a slightly raised serum phosphatase in dairy cows, though the level of phosphatase does not appear to be correlated with the stage of pregnancy.

6. No evidence was found of correlation between milking capacity and serum phosphatase activity in dairy cows.

7. The high phosphatase activity of the sera of some clinically healthy cows does not appear to be due to an enzyme activator in these sera.

Our best thanks are due to Prof. H. D. Kay and Dr H. H. Green for their supervisory interest in this work. We are also deeply grateful to all those, too numerous to name here, who by their generous co-operation have made it possible to obtain blood samples. Mrs Ruth Allcroft kindly carried out a number of phosphatase determinations, while the technical assistance of Messrs F. J. Salt and S. C. Watson was greatly appreciated.

REFERENCES

Auchinachie & Emslie (1933). *Biochem. J.* **27**, 351.
Bodansky (1933). *J. biol. Chem.* **101**, 93.
—— (1934). *J. biol. Chem.* **104**, 717.
Folley (1936). *Biochem. J.* **30**, 2262.
—— & Kay (1936, 1). *Ergebn. Enzymforsch.* **5**, 159.
—— —— (1936, 2). *Enzymologia*, **1**, 48.
—— & White (1936). *Proc. roy. Soc.* B, **120**, 346.
Gardner & Pfeiffer (1938). *Proc. Soc. exp. Biol., N.Y.*, **37**, 678.
Gutman & Gutman (1940). *J. biol. Chem.* **136**, 201.
Kay (1930, 1). *J. biol. Chem.* **89**, 244.
—— (1930, 2). *J. biol. Chem.* **89**, 253.
King & Armstrong (1934). *Canad. med. Ass. J.* **31**, 376.
—— & Delory (1938). *Biochem. J.* **32**, 1157.
Lyle Stewart (1933). *Vet. J.* **89**, 63.
McCollum & Hart (1908). *J. biol. Chem.* **4**, 497.
Mulay & Hurwitz (1938). *J. Lab. clin. Med.* **23**, 1117.
Piercy (1934, 1). *Vet. J.* **90**, 53.
—— (1934, 2). *Vet. J.* **90**, 223.
Scoz & Marangoni (1934). *Boll. Soc. ital. Biol. sper.* **9**, 969.
Smith & Maizels (1932). *Arch. Dis. Childh.* **1**, 149.
Stearns & Warweg (1933). *J. biol. Chem.* **102**, 749.
Thannhauser, Reichel & Grattan (1937, 1). *J. biol. Chem.* **121**, 697.
—— —— —— (1938, 1). *Biochem. J.* **32**, 1163.
—— —— —— & Maddock (1937, 2). *J. biol. Chem.* **121**, 709.
—— —— —— —— (1938, 2). *J. biol. Chem.* **124**, 631.
Vermehren (1939). *Acta. med. scand.* **100**, 254.
Wilson & Hart (1932). *J. Dairy Sci.* **15**, 116.

27. THE DETERMINATION OF THE VISCOSITY OF HUMAN MILKS AND THE PRENATAL SECRETIONS

By GEORGE WILLIAM SCOTT BLAIR

From the National Institute for Research in Dairying,
University of Reading

(*Received 20 January 1941*)

THAT the marked variations which are known to exist in the viscous properties of human mammary secretions are of interest in studying both normal and pathological conditions has been pointed out by Waller [Waller *et al.* 1941]. In order to assist in a further investigation of the question it seemed advisable to devise a viscosimeter suitable for the study of such materials and to establish the general character of their rheological properties.

In the case of prenatal secretions removed from the breast by manipulation, it is frequently the case that only about a millilitre of material is available for the viscosity measurement, nor is the viscosity always a constant independent of shearing stress. A viscosimeter must therefore be designed which will give a series of readings on a very small sample at a number of different shearing stresses. The instrument shown in Fig. 1 is in principle quite similar to several others already described in the literature, especially by Ostwald and his coworkers, but is designed as the most suitable modification for the present purpose.

The apparatus consists of a glass capillary tube (*A*) sealed into two wider tubes but at right angles so that the whole can be immersed in a thermostat water bath. Approximately 1 ml. of the material to be tested is run by means of a pipette into the wide mouth *B* and allowed to stand for a few moments to reach temperature equilibrium and to find its level on the two sides of the viscosimeter. If there is much 'creaming' this must be done as quickly as possible, the milk being brought approximately to temperature previously in another vessel. A temperature of 37° is used, partly because this is the natural temperature for such materials and partly because it has been shown in the case of cow's milk (especially cream) by Scott Blair *et al.* [1939] that additional rheological complications are introduced when temperatures are used at which the fat phase is liable to solidify.

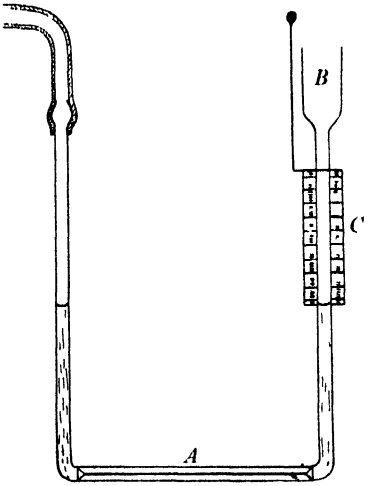


Fig. 1.

The sliding scale (*C*) is then adjusted so that the zero of the scale corresponds to the meniscus of the sample.

The sample is then blown into the tube by means of a rubber tube attached to the other arm. When the pressure is released, the column of liquid starts to fall along the scale (C). When the meniscus passes a convenient point on the scale (h_1) (18 cm. is usually suitable) a stop-watch is started and the time taken for the meniscus to fall to half this height (h_2) is recorded (t_1). The test is then repeated starting at h_2 and timing the fall to h_3, a distance half that of h_2, i.e. a quarter of the original h_1. This second time t_2 should be equal to t_1 if the material is a true fluid obeying Poiseuille's law. If the material is not a true fluid, the ratio of t_1 to t_2 is an index of the extent of the anomaly. The viscosity in C.G.S. units (poises, or better, the kinematic viscosity of which the unit is the stokes, and which is equal to the viscosity/density) is best derived by standardizing the instrument by means of sucrose solutions of known viscosity, and a number of instruments having capillaries of different radii should be used conveniently to cover the whole range of human mammary secretions. A constant is thus obtained for each instrument from which viscosities can be worked out in C.G.S. units (poises or stokes) by multiplying by the value of t_1. Where the viscosities differ for the two ranges of stress, that calculated from a fall from $h_1 = 18{\cdot}0$ to $h_2 = 9{\cdot}0$ is arbitrarily chosen. The errors involved in this choice of stress are small compared with the range of viscosities involved. This simple test takes only a few moments to complete, and the result is calculated by means of a single multiplication.

The above treatment is derived from the following considerations, which also serve to give an absolute standardization of the instrument if such is desired. For materials differing appreciably from truly fluid behaviour, or where only very small samples are available, the direct application of the equation now to be derived is necessary.

Let V be flow rate of sample through capillary in cm.^{3}/sec.,
R_c be radius of capillary (cm.),
P be pressure produced by head h at time t sec., in dynes/cm.^{2},
L be length of capillary tube (cm.),
η be viscosity and ρ the density of sample,
R_w be radius of wide tube (cm.) (assumed large compared with R_c),
v be velocity of meniscus falling in wide tube (cm./sec.).

From Poiseuille's law we have

$$V = \frac{P\pi R_c^4}{8L\eta}$$

(assuming that the viscous resistance in the wide tube is negligible),

or
$$v = \frac{PR_c^2}{8L\eta} \times \frac{R_c^2}{R_w^2} = \frac{PR_c^4}{8L\eta R_w^2}.$$

The rate of decrease of head is twice this, since the meniscus falls on one side but rises at an equal rate on the other; hence

$$\frac{dh}{dt} = -\frac{h\rho g R_c^4}{4L\eta R_w^2},$$

where g is the gravity acceleration,

or
$$t = -\frac{4L\eta R_w^2}{\rho g R_c^4} \int \frac{1}{h}\, dh = -\frac{4L\eta R_w^2}{\rho g R_c^4} \log_e h + C,$$

where C is an integration constant.

Now, when $t = 0$, we have $h = h_0$, hence

$$C = \frac{4L\eta R_w^2}{\rho g R_c^4} \log_e h_0;$$

hence η/ρ, the kinematic viscosity, which we will write ν, is given by

$$\eta/\rho = \nu = \frac{t}{\log_{10}(h_0/h)} \frac{gR_c^4}{4LR_w^2} 0{\cdot}4343.$$

Thus, if we are prepared to discount differences in the densities of the samples, i.e. to use kinematic rather than dynamic viscosities, we see that the slope of the curve plotting t against $\log_{10}(h_0/h)$ has only to be multiplied by a constant depending on the dimensions of the apparatus to give the kinematic viscosity in absolute units (stokes). In view of the range of viscosities met with, it is convenient to multiply these figures by 100 and to quote ν in centistokes.

A convenient range of values for R_c is from about 0·03 to 0·08 cm. R_w is conveniently about 0·25 cm. and L about 20 cm.

For most materials the fixing of h_0/h at 2·0 as described in the technique proposed above avoids the trouble of plotting any graphs, but where samples are very meagre, or very anomalous in behaviour, the $t/\log h_0/h$ curve should be plotted. An empirical figure representing the extent of the viscous anomaly is then conveniently obtained by plotting logs of both expressions, the extent to which the slope of the log-log curve differs from unity being a measure of the extent of the viscous anomaly. (For further explanation, see Scott Blair [1938].)

Table 1. *Variation of $t/\log h_0/h$ with time, for water*

t (sec.)	2	4	6	8	10	12	14	16	18	20	22
$t/\log h_0/h$	23·2	21·6	21·3	21·5	22·0	21·8	21·8	21·9	21·8	21·8	21·1

Table 2. *The effect of progressive shearing and resting on $t/\log h_0/h$ for human colostrum*

	t (sec.)	2	4	6	8	10	12	14	15	16	18	20	22	24
Shear No.	1	250	266	286	276	277	279	280	—	271	265	267	275	282
	2	—	235	—	242	—	266	—		271	—	278	—	282
	3		—	286	—	—	279	—	—	—	272	—	—	289
	4	—	—		—	—	—	—	263	—	—	—	—	—
	5*		—	—	—	—	—	—	—	—	—	—	—	—
	t (sec.)	26	28	30	32	34	36	38	40	42	44	45	46	48
Shear No.	1	279		280	—	278		280		282	—	—	270	—
	2	—	277		278	—	283	—	280	—	286	—	—	282
	3	—	—	297	—	—	290	—		282	—	—	—	288
	4	—	—	270	—	—	—	—	—	—	—	281	—	—
	5*	—	—	263	—	—	—	—	—	—	—	—	—	—
	t (sec.)	50	52	54	56	60	64	66	68	72	75	76	78	80
Shear No.	1	274	—	—	—	—	—	—	—	—	—	—	—	—
	2	—	278	—	280	279	281	—	282	287		284	—	285
	3	—	—	289	—	290	—	290	—	296	—	—	291	—
	4	—	—	—	—	276		—	—	—	281	—	—	—
	5*	—	—	—	—	267	—	—	—	—	—	—	—	—
	t (sec.)	84	88	90	96	102	105	108	114	120	126	132	135	138
Shear No.	1	—	—	—	—	—	—	—	—	—	—	—	—	—
	2	287	292	—	—	—	—	—	—	—	—	—	—	—
	3	291	—	290	292	293	—	294	290	294	294	293	—	295
	4	—	—	283	—	—	281	—	—	285	—	—	289	—
	5*	—	—	278	—	—	—	—	—	281	—	—	—	—
	t (sec.)	144	150	156	165	180	195	210	225	240	255	270	285	300
Shear No.	1	—	—	—	—	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—	—	—	—	—
	3	299	299	294	—	—	—	—	—	—	—	—	—	—
	4	—	291	—	290	294	296	296	297	297	294	294	297	300
	5*	—	283	—	—	290	—	296	—	296	—	311	—	313

* After standing for 1¼ hr., having been gently stirred after ¾ hr.

EXPERIMENTAL

As a check on the accuracy of one of these viscosimeters an experiment was done with pure distilled water. The values of $t/\log h_0/h$ are given in Table 1. They are satisfactorily constant. Some materials show a tendency for the viscosity to fall as a result of repeated shearing [Scott Blair *et al.* 1939]. This makes it hard to decide on any unique value for viscosity. In some cases, the viscosity is partially recovered on standing (thixotropy or false-body). It has been shown (Table 2) that these properties are not present to any marked extent in the case of normal human mammary secretions. Progressive shearing and resting are seen to have little or no effect on this flow curve for a colostral milk sample.

It is not intended to publish here any large number of data or to discuss their bearing on clinical problems. This is being done in another article [Waller *et al.* 1941]. A few typical curves only will be given. These are shown in Fig. 2.

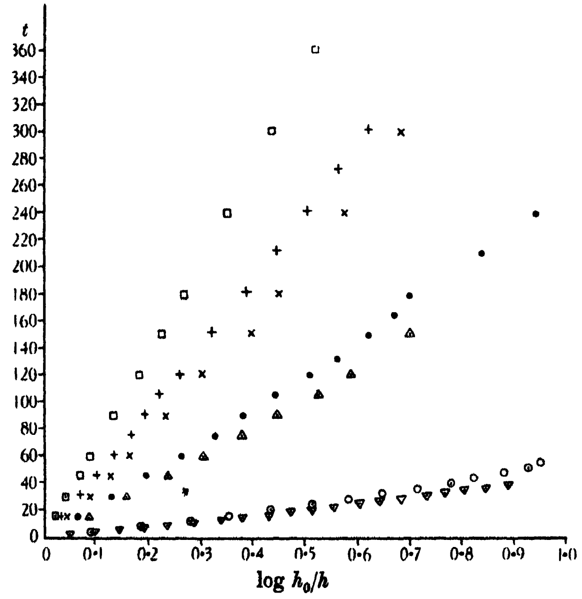


Fig. 2.

	No.	$t/\log h_0/h$	η	Notes
⊙	14	46	1·44	5th day milk, 2nd para.
×	15	394	12·35	38 weeks (about due), 1st para.
△	18	204	6·40	38 weeks, 1st para.
□	20	668	20·8	Just after delivery, 1st para.
+	21	470	14·7	3rd day milk, 1st para.
▽	22	42	1·31	4th day milk (engorgement), 1st para
●	23	236	7·38	38 weeks, 1st para.

It will be seen that the curves are very nearly but not quite linear (a few samples showed even more marked anomalies), and that the viscosities varied over a wide range. Although no such extreme samples appear in the figure, values as high as 100 centistokes have been obtained for prenatal secretions, compared with an average of about 1·3 centistokes for normal milks.

The fall in viscosity with rising shearing stress is gradual. There is no sign of a yield value, which leads to the conclusion that all these secretions would flow through any uncontrolled orifice even at very low pressure. The prevention of seepage thus depends on the behaviour of the ducts rather than on the properties of the secretion even when viscosity is very high.

SUMMARY

1. A method is described by which the viscosity and viscous anomalies of small (1 ml.) samples of human mammary secretions may be quickly and accurately determined.

2. These secretions, tested at blood heat, behave in general surprisingly like true fluids, i.e. their viscosities differ but little with varying shearing stress. The viscosities themselves vary over a very wide range—at least a hundredfold.

3. Where viscous anomalies exist, they appear as a gradual fall in viscosity with rising stress, suggesting that even the most viscous materials would flow slowly under extremely small pressures.

REFERENCES

Scott Blair (1938). An Introduction to Industrial Rheology. London: Churchill.
—— Hening & Wagstaff (1939). *J. phys. Chem.* **43**, 853.
—— Aschaffenburg & Grant (1941). *Biochem. J.* **35**, 272.

28. THE VISCOSITY, PROTEIN DISTRIBUTION, AND 'GOLD NUMBER' OF THE ANTENATAL AND POSTNATAL SECRETIONS OF THE HUMAN MAMMARY GLAND

BY HAROLD WALLER, RUDOLF ASCHAFFENBURG AND
MARGARET WILSON GRANT

From the British Hospital for Mothers and Babies, Woolwich, and from the National Institute for Research in Dairying, University of Reading

(*Received 20 January 1941*)

IN his *Clinical Studies in Lactation* Waller [1938] has discussed the manifold problems and difficulties connected with the onset of lactation and has, in particular, drawn attention to the frequency of engorgement with its serious consequences to the maintenance of secretion, and to the possibility of reducing this frequency by the systematic removal of the prenatal breast secretion. It was then pointed out that 'it would be extremely helpful if we knew the relative viscosity of the three types of fluid (prenatal secretion, colostrum and milk) which leave the breast and whether...viscosity bears a direct relation to the protein content'. A suitable method for the measurement of kinematic viscosity was, therefore, developed by Scott Blair [1941], the viscosity of these secretions having so far only been qualitatively assessed [Widdows *et al.* 1935]. It was intended to correlate the viscosity data with the total protein content and, at the same time, to determine some of the major protein fractions, such as globulin and casein. It was, however, soon realized that analytical methods have not been established for the accurate determination of all these constituents in fluids varying so widely in composition as the antenatal and postnatal human secretions. Whilst we were unable to undertake the fundamental investigations obviously needed to provide such methods, we have collected a number of nitrogen partition data of comparative value sufficient to show the approximate levels and changes during the antenatal and early postnatal periods. The viscosity data, however, were correlated with the indisputable total nitrogen figures. In addition, a rapid and simple gold sol reaction was developed which reflects closely the changes in the total nitrogen level of the secretions.

As far as was practicable, samples from each woman included in this investigation were tested on several occasions during pregnancy and daily for the first week or so after parturition. The research thus covers, in part, the same field as that of Lowenfeld *et al.* [1927] and Widdows *et al.* [1935]. It should be pointed out, however, that in the present investigation the prenatal secretion of the subjects was as a rule regularly expressed from the breast. By obtaining results on a relatively large range of material we endeavoured, apart from the establishment of the relationship between viscosity and total nitrogen, to find out whether the regular removal of the prenatal secretion causes marked changes in the physical or chemical make-up of the fluid as it does in the cow [Woodman & Hammond, 1922; 1923; Asdell, 1925]; whether such removal leads to a shortening of the colostral period (for which purpose a number of 'control' cases was included in which no expression had been practised before childbirth); and whether engorgement exerts an influence on the physical behaviour or the chemical composition of the postnatal secretion.

METHODS

With prenatal samples the total volume obtainable by manual expression was used for analysis. With postnatal samples it was not found practicable to apply a uniform method of sampling. Portions of the secretion were obtained either by manual expression or by application of the breast-pump. The samples were taken direct to the laboratory and analysed immediately, or when this was not possible were stored in a refrigerator. Estimations on stored samples at daily intervals showed that no change in viscosity took place during 4 days and that the total nitrogen remained unchanged for 60 hr., though there was a slight rise in non-protein nitrogen.

Storage at room temperature for 3 days did not affect the 'gold number'. This was important, as the samples required for the gold test had to be sent through the post.

Viscosity. The method is described in a separate paper [Scott Blair, 1941].

Nitrogen partition. (*a*) Total nitrogen was determined by micro-Kjeldahl analysis, using 2 ml. of suitably diluted secretions. No standard dilution could be adopted, as the concentration of the samples varied so widely. The viscosity readings provided a useful basis for determining the amount of dilution required in each case.

(*b*) Non-protein nitrogen. Numerous precipitating agents have been recommended for physiological fluids. While giving comparative values, they differ in action, bringing down varying amounts of proteose-peptone compounds in addition to the 'true' proteins. Several of these reagents were tried, but difficulties were encountered with all of them, a number of samples, both ante- and postnatal, giving cloudy filtrates. Pending the development of adequate methods we adopted precipitation with strong trichloroacetic acid, as this reagent seems to provide the nearest approach to the precipitation of the 'true' proteins only [cf. Seibert, 1926].

(*c*) Casein. An adaptation of the method recommended for cow's milk by Rowland [1938] was used. It was found necessary to reduce the amounts of precipitating agents so as to ensure precipitation of the smaller amounts of casein present in human secretions at a *p*H in the neighbourhood of the isoelectric point. Suitable conditions were determined by varying the concentration and proportions of acetic acid and sodium acetate in conjunction with *p*H measurements (glass electrode). The following procedure was adopted for milk: 1 ml. of milk is diluted with *ca*. 15 ml. of water at 40°, 1 ml. of $N/10$ acetic acid is added, followed after 10 min. by 0·8 ml. $N/10$ Na-acetate solution. After cooling, the volume is made up to 20 ml. Rowland's procedure is then followed in detail. The more concentrated prenatal and early postnatal samples were suitably diluted so that the nitrogen content approximated to that of normal milk. Apart from other considerations, this course was necessitated by the fact that often no more than 1 ml. of secretion was available for determining the nitrogen partition.

(*d*) Globulin was determined in the casein-free filtrate as described by Rowland [1938].

'Gold number'

The 'gold number' (Zsigmondy) is defined as the weight of a given protecting colloid just insufficient to prevent a change of colour in 10 ml. of a stable red gold sol when 1 ml. of 10 % solution of NaCl is added to it. For the purpose of this investigation the 'gold number' of the ante- and post-natal secretions which contain a mixture of protecting colloids has been defined as the reciprocal of the dilution of secretion contained in 1 ml. of solution which is just insufficient to prevent a change of colour in 5 ml. of a stable red gold sol when 0·5 ml. of 10 % solution of NaCl is added to it. It appeared preferable to express the 'gold number' as the reciprocal of the dilution, as in this way a higher 'gold number' corresponds to a higher protecting power of the secretion.

Many workers have encountered difficulties in the routine preparation of gold sols. The technique described by Pennycuick *et al.* [1938] was found to be free from the usual hazards and permitted the preparation of sols of the desired type with ease and regularity. The *p*H of the sols requires no adjustment. Fresh sols should be prepared every 6–8 weeks, as it was noted that after this interval the sols tend to become slightly purplish and to lose their sensitivity.

When dealing with prenatal secretions and early colostrum a series of aqueous dilutions of 1 : 1000, 1 : 2000, 1 : 3000, ... is made up in test tubes. Dilution 1 : 1000 is prepared by diluting 0·1 ml. of the fluid to be tested to 100 ml. using a 0·1 ml. wash-out pipette.

When dealing with later colostrum and milk, dilutions of 1 : 250, 1 : 500, 1 : 750, 1 : 1000, ... are similarly prepared, first making up 1 ml. of fluid (or 0·2 ml. where little material is available) to 100 ml.

1 ml. of each dilution is then pipetted into a wide hard-glass test tube (5 × $\frac{5}{8}$ in. is a convenient size) and 5 ml. of gold sol are rapidly run in with shaking, followed after 10 min. by 0·5 ml. of a 10 % solution of NaCl. The interval between the addition of the gold sol and that of the NaCl solution is not without influence on the result of the test. The period of 10 min. was chosen as being convenient when dealing with several series of samples. Readings are taken after 1 hr., the reciprocal of the first dilution showing a distinct colour change to red-violet being noted as the 'gold number' of the fluid under test. The gold sol in all tubes containing higher concentrations assumes a faint purplish tinge on addition of the NaCl. This uniform change should be disregarded. Duplicate tests were found to agree closely, and with some experience it is often possible to interpolate between the readings of two successive tubes of the series given above. Good agreement was observed when different batches of gold sol were used for the testing of the same dilution series. Cases are encountered in which the 'gold number' drops below 500, and the turbidity of the milk is then apt to interfere to some extent with the colour readings. In such cases useful information is obtained by recording the colour change in the gold sol produced by a 1 : 1000 dilution of the sample.

RESULTS

Viscosity, total nitrogen and gold number

(*a*) *Prenatal secretions*

Most specimens were obtained during the last 8–10 weeks of pregnancy. In a few cases it was possible to secure earlier samples; these showed no unusual properties.

The samples varied from a colourless, sticky and apparently fat-free fluid to a rich yellow, creamy mixture from which a thick layer of fat readily separated out on standing.

Space does not permit us to give the results of the serial examinations in detail. They may be summed up as follows: whilst the variations in viscosity, total nitrogen and gold number in individual cases observed at intervals during late pregnancy are considerable, none of these factors shows a systematic decrease or increase as a consequence of the regular removal of the secretion. There is, however, a tendency in many, though not in all, cases for these factors to attain maximum values at the time of, or shortly after, parturition.

The range of values is set out in Tables 1–3, in which only the result of the first examination is included in the many cases in which specimens were repeatedly tested. The tables clearly show the peaks in the frequency of distribution with no particular difference between primiparae and multiparae.

Table 1. *Viscosity of prenatal secretions from 45 women (centistokes)*

	<5	5–15	15–25	>25
27 primiparae	5	17	2	3
18 multiparae	4	11	1	2

Table 2. *Total nitrogen of prenatal secretions from 30 women (g./100 ml.)*

	0–0·5	0·5–1·0	1·0–1·5	1·5–2·0	2·0-2·5	2·5–3·0
18 primiparae	0	3	10	2	3	0
12 multiparae	0	1	9	1	0	1

Table 3. *Gold number of prenatal secretions from 40 women other than those included in Tables 1 and 2*

Gold number	<1000	1000	2000	3000	4000	5000	6000
23 primiparae	0	0	2	12	6	2	1
17 multiparae	1*	0	2	3	6	2	3

* Pathological fluid, patient suffering from severe abscesses.

The gold numbers of specimens from both breasts of the same subject were determined in a number of cases. No marked differences were found, even where differences were noted in the ease of expression, quantity or appearance of the fluids.

(*b*) *Postnatal secretions*

(α) *During the first days of lactation.* The results obtained by following a number of cases through the early stages of lactation proper are summed up in Tables 4–6. They show that the viscosity, total nitrogen and gold number all fall rapidly to new and much lower levels during the first 5 days *post partum.* The initial levels vary greatly, as is indicated by the highest and lowest values given in the tables, but individual cases which cannot be given *in extenso* show a fairly uniform behaviour, namely, a sharp drop from the high to the low level requiring usually between 24 and 48 hr. only. This drop starts in the majority of cases on the 2nd or 3rd day *post partum,* sometimes as early as a few hours after delivery and sometimes as late as the 4th day after parturition.

Lowenfeld *et al.* [1927] followed the protein level in a relatively small number of cases and report a marked difference between primiparae and multiparae, the latter attaining the low level earlier than the former. Examination of the total nitrogen level of a similarly small number of cases which had received no treatment before delivery provided little confirmatory evidence of this observation.

Table 4. *Viscosity of postnatal secretions from 33 women* (*centistokes*)

22 primiparae

Days *post partum*	1*	2	3	4	5	6	7	8	9
Mean	13·95	9·91	5·26	2·07	1·48	1·44	1·34	1·39	1·36
Highest	51·90	25·10	20·00	5·05	1·77	1·80	1·56	1·62	1·66
Lowest	3·11	1·96	1·18	1·24	1·18	1·18	1·13	1·18	1·13

* 7 cases only.

11 multiparae

Days *post partum*	1*	2	3	4	5	6	7	8	9
Mean	10·18	14·11	4·51	1·59	1·36	1·46	1·37	1·38	1·32
Highest	23·10	43·00	26·40	1·86	1·58	1·94	1·63	1·84	1·47
Lowest	2·23	2·23	1·56	1·45	1·24	1·24	1·18	1·13	1·18

* 6 cases only.

Table 5. *Total nitrogen of postnatal secretions from 27 cases* (*g./100 ml.*)

20 primiparae

Days *post partum*	1*	2	3	4	5	6	7	8	9
Mean	1·25	1·37	1·42	0·73	0·41	0·37	0·35	0·29	0·33
Highest	1·83	2·00	3·47	1·48	0·67	0·51	0·39	0·43	0·39
Lowest	0·89	0·46	0·32	0·32	0·31	0·28	0·28	0·28	0·29

* 5 cases only.

7 multiparae

Days *post partum*	1*	2	3	4	5	6	7	8	9
Mean	1·70	2·02	0·69	0·59	0·34	0·38	0·34	0·31	0·29
Highest	1·71	3·07	0·82	1·24	0·36	0·48	0·38	0·36	0·34
Lowest	1·69	1·39	0·59	0·34	0·31	0·29	0·28	0·27	0·26

* 2 cases only.

Table 6. *Gold number of postnatal secretions from 38 women*

20 primiparae

Days *post partum*	1	2	3	4	5	6	7	8
Mean	4000	3500	2000	1200	800	800	800	800
Highest	6000	5000	3500	4000	1000	1500*	1500*	1000
Lowest	2000	1500	750	750	500	500	500	500

* 1 case only, all others 1000 or less.

18 multiparae

Days *post partum*	1	2	3	4	5	6	7	8
Mean	4500	4000	1800	1100	800	800	750	800
Highest	6000	8000	3000	1750	1250	1000	1000	1000
Lowest	3000	1750	1250	750	750	500	500	500

No difference was found in the behaviour of untreated cases and of those in which the prenatal fluid had been regularly removed. All cases were, therefore, included in the tables.

As with prenatal secretions, the gold number of samples from both breasts was determined in a number of cases. Here again the results were identical almost without exception, even during the first few days *post partum* with their rapid and drastic changes. Special attention was paid to the incidence of engorgement, which occurred in a more or less severe form in many instances. No evidence was found of any effect of this disturbance on the factors under discussion. The significance of this finding is discussed below.

(β) *During later lactation.* Few cases were followed up regularly beyond the first 10 days or so after parturition, as most patients became unavailable on leaving the hospital. In any case, many data on the total nitrogen level for the ensuing period of lactation are already to be found in the literature [cf. Widdows *et al.* 1930; Bell, 1928], showing that the total nitrogen falls very slowly as lactation progresses. Measurements of the viscosity and the gold number showed that the same applies to these factors. In order to obtain a more sensitive measure of the protecting power under these conditions, the procedure described above of reading the colour of the gold sol in a standard milk dilution of 1 : 1000 was reverted to. All samples obtained between the 5th and 22nd weeks of nursing in cases in which lactation was progressing satisfactorily gave colour changes to blue or bluish violet, whilst the maximum change of colour during the first fortnight *post partum* was one to violet, a reddish violet being the rule.

The relation between viscosity and total nitrogen

By plotting viscosity (ν) against concentration of total nitrogen (c) it was found that these factors are not connected by a simple, linear relationship, the viscosity rising rather more steeply than the concentration of nitrogenous compounds. This is not surprising, considering that Einstein's [1906] equation[1] $\nu = k.c + b$ is based on the assumption of uncharged spherical particles of constant hydration. By plotting log viscosity against the concentration of total nitrogen a straight-line function was obtained, the majority of data fitting the equation

$$\log \nu = 0{\cdot}65.c - 0{\cdot}07$$

reasonably well. Some of the more viscous fluids, however, showed considerable deviations, so that the above equation should not be regarded as more than a convenient generalized expression. No more could be expected considering that with the complex colloidal systems under discussion not only the amount but also the state of dispersion of the colloidal particles will doubtless exert its influence, apart from the possible effect of the fat concentration on the viscosity, which appears, however, to be of secondary importance. That the equation holds in a general way is shown in Table 7, in which the mean values for total nitrogen as calculated from the viscosity data given in Table 4 (primiparae and multiparae combined) are set out against the corresponding total nitrogen values given in Table 5.

Table 7. *Relation between viscosity and total nitrogen*

Days *post partum*	1	2	3	4	5	6	7	8	9
Total N calculated from ν	1·8	1·8	1·2	0·5	0·35	0·35	0·3	0·3	0·3
Total N calculated from Table 5	1·5	1·7	1·05	0·65	0·35	0·4	0·35	0·35	0·3

The relation between gold number and total nitrogen

Plotting the gold number (G) against corresponding values of total nitrogen concentration (c), a straight-line function was obtained and calculated to

$$G = 3000.c - 200.$$

---

[1] Einstein's equation was developed for dynamic and not kinematic viscosity, but it seems unlikely that density differences will be great enough to make this distinction significant.

[2] The utility of applying an equation of this type—probably first used by Arrhenius—has been demonstrated by Scott Blair *et al.* [1927], who employed it successfully when dealing with flour suspensions.

With few exceptions this equation was found to hold well within the limits of accuracy of the gold sol reaction. To demonstrate the close agreement obtained the cases in which both the total nitrogen and the gold number were determined simultaneously during the first few days after parturition are summarized in Table 8. The simple gold sol reaction may, therefore, be used to advantage in place of the more tedious Kjeldahl determinations when it is intended to follow the major changes during the prenatal and early postnatal stages of secretion.

Table 8. *Relation between gold number and total nitrogen*

Days *post partum*	2	3	4	5	6	7	8
Total N, mean	1·64	0·81	0·54	0·38	0·35	0·34	0·32
Total N calculated from the gold number	1·54	0·87	0·57	0·36	0·33	0·35	0·33

Nitrogen partition

For the reasons already stated only a limited number of data have been collected.

(*a*) *Non-protein nitrogen and total protein*

Table 9 contains a summary of the results.

Table 9. *Non-protein nitrogen* (*mg./100 ml.*)

	Mean	Highest	Lowest
15 prenatal secretions	105	200	69
7 postnatal secretions 1–4 days *post partum*	91	127	51
21 postnatal secretions 5–14 days *post partum*	84	140	48

As compared with the total nitrogen, the non-protein nitrogen falls but little during the first few days after parturition. No strictly comparable data are available in the literature. A few values for colostrum and early milk obtained by precipitation with tungstic acid are given by Erickson *et al.* [1934]. Their non-protein nitrogen values are markedly lower and this would seem to indicate the presence of considerable amounts of proteose-peptone compounds in our trichloroacetic acid filtrates. Further investigations in this direction are desirable.

When viewing the non-protein nitrogen data in conjunction with the corresponding total nitrogen figures, it was found that during the prenatal period the non-protein nitrogen constitutes a small and fairly constant proportion of the total nitrogen. During early lactation, however, the percentage of non-protein nitrogen increases markedly, as is shown in Table 10.

Table 10. *Non-protein nitrogen as percentage of total nitrogen*

	%		
No. of cases	0–15	15–30	30–45
Before parturition	15	0	0
1–4 days *post partum*	6	1	0
5–14 days *post partum*	0	15	6

It must be concluded that after the first few days of lactation one is hardly justified in regarding the total nitrogen as a fair representation of the total protein. There is little doubt that the latter continues to fall slowly after 4 days of lactation, whilst the former appears to remain almost constant (see Table 5). This is borne out by the findings of Lowenfeld *et al.* [1927].

(b) *Casein nitrogen*

Casein was found to be present in considerable amounts as early as the 22nd week of pregnancy. Up to the time of delivery its concentration changes roughly in proportion to that of total nitrogen; it then shows a similar, if less pronounced, drop during the first 4–5 days of lactation, after which it changes little up to the 14th day *post partum*. The results are summarized in Table 11.

Table 11. *Casein nitrogen (mg./100 ml.)*

	Mean	Highest	Lowest
43 prenatal secretions	334	705	100
40 postnatal secretions 1–4 days *post partum*	323	812	114
84 postnatal secretions 5–14 days *post partum*	144	289	64

When the amount of casein is considered as a percentage of the total nitrogen, a definite increase is noted as lactation is established (see Table 12).

Table 12. *Casein nitrogen as percentage of total nitrogen*

	%				
No. of cases	10–20	20–30	30–40	40–50	>50
Before parturition	17	13	12	0	1
1–4 days *post partum*	4	15	15	5	1
5–14 days *post partum*	1	5	27	34	17

(c) *Globulin nitrogen*

Globulin was found to be the major protein constituent of the prenatal secretions. During advanced pregnancy its concentration varies, in general, in the same manner as that of total nitrogen. After parturition the globulin continues to behave similarly to the total nitrogen, dropping sharply within the first 4–5 days *post partum*. This drop is, however, followed by a further slow decrease up to 10–12 days after delivery.

The results are summed up in Tables 13 and 14.

Table 13. *Globulin nitrogen (mg./100 ml.)*

	Mean	Highest	Lowest
41 prenatal secretions	825	1896	281
39 postnatal secretions 1–4 days *post partum*	556	2080	66
83 postnatal secretions 5–14 days *post partum*	78	214	33

Table 14. *Globulin nitrogen as percentage of total nitrogen*

	%	
No. of cases	0–40	40–80
Before parturition	2	39
1–4 days *post partum*	13	26
5–14 days *post partum*	80*	3

* Mostly 10–30 %.

(d) *The ratio globulin/casein*

Perhaps the most characteristic difference between the antenatal and postnatal secretions is seen in the ratio globulin/casein as set out in Table 15.

Table 15. *Globulin/casein ratio*

	Ratio	
No. of samples	>1·0	<1·0
Before parturition	37	2
1 day *post partum*	4	1
2 days *post partum*	8	0
3 days *post partum*	9	1
4 days *post partum*	6	7
5–14 days *post partum*	7	76

DISCUSSION

Woodman & Hammond [1922; 1923] and Asdell [1925] have shown that the regular removal of the prenatal secretion of the cow or heifer causes drastic changes in the physical and chemical properties of the fluid as pregnancy progresses and leads to a state simulating lactation proper. The results presented here indicate that in the human subject the effects of such removal are fundamentally different. The protein make-up and the viscosity of the fluid, though subject to variations, do not alter systematically and there is nothing to indicate that regular expression stimulates the production of an increased amount of a more milk-like fluid. It seems significant that in the human subject the ratio globulin/casein remains high until some time after parturition, whereas in the case of the heifer studied by Asdell this ratio fell below 1·0 as early as the 32nd week of pregnancy.

In agreement with Widdows *et al.* [1935] we found that the prenatal secretions fell into three distinct classes, the appearance of each being unconnected with the state or number of pregnancies. Our attempts to gauge the viscosity from the appearance of the secretion in the manner described by Widdows *et al.* failed, the result of the actual measurement being often in disagreement with the visual judgement. That these authors were, nevertheless, able to predict a definite relation between the viscosity and the protein content of the secretions may be explained by the fact, brought out in our investigations, that small variations in the protein content correspond to large changes in viscosity, the total nitrogen (and, therefore, presumably the total protein) being connected by a linear function with the logarithm of viscosity and not with the viscosity itself.

Turning to the postnatal stages, it seems necessary, first of all, to remark briefly upon the term 'human colostrum'. The uncertainties attaching to this term have been fully discussed by Lowenfeld & Widdows [1928] and by Waller [1938]. Here it suffices to point out that whilst it is true that the human secretion continues to differ in some respects from 'mature' milk up to the end of the first month of lactation, a fluid essentially similar to this 'mature' milk is already being secreted after a few days of lactation only. Is it unreasonable to suggest that the term 'colostrum' should not be applied to this milk-like fluid, but that its use should rather be confined to the fluid fundamentally different from milk which is produced during the first few days after parturition? The results presented above seem to us to favour this suggestion. They show that measurable criteria of both a physical and a chemical nature are available, viz. the viscosity, the total nitrogen content and the globulin/casein ratio, all pointing to the conclusion that the time during which 'colostrum' is secreted is short and does not, as a rule, exceed a period of more than 5 days after delivery. Of these criteria the viscosity and the total nitrogen, or—in its place—the gold number, lend themselves most readily to measurement, but the ratio globulin/casein is perhaps of greatest significance, as it reflects a fundamental change from the predominance of globulin to that of casein. It is to be hoped that more satisfactory methods for the determination of these protein fractions will soon become available.

Contrary to Lowenfeld *et al.* [1927], we found no difference between the duration of the colostral stage in primiparae and multiparae in untreated cases. Neither was there any evidence that the regular removal of the antenatal secretion shortens the colostral period, and this fact, taken in conjunction with the observations made on the prenatal fluids, speaks strongly against the conception of stimulation of the lactiferous glands by this treatment.

It has been shown that the transition from colostrum to 'early milk' does not, in general, require more than 24–48 hr. It seems likely that the starting time depends on the time required by the body to set in motion the hormonal functions controlling lactation proper and that, once these have begun to work, milk rapidly replaces colostrum. If to this is added the observation that engorgement has no demonstrable effect on the physical or chemical nature of the fluid within the breast, one is driven to the conclusion that the mechanism of milk production is established with truly remarkable regularity and that it is the mechanism of expulsion rather which is so frequently at fault. It follows that, contrary to expectations at the outset of this investigation, a study of the composition and properties of the secretions cannot be of help in explaining the beneficial effect of the prenatal treatment which has been established beyond doubt by clinical observations at the British Hospital for Mothers and Babies. The causes of engorgement must be sought rather in factors connected with the mechanism of outflow. Whether the prevention of engorgement by prenatal treatment is due to a simple 'training' effect or to specific anatomical, physiological or psychological factors remains to be seen. As engorgement presents itself as the consequence of 'a gland functioning actively against obstruction to outflow' (Waller), future investigations must clearly be directed towards elucidation of the nature of the hindrance. A promising line of approach is suggested by the interesting study of Seitz [1924–25], whose work on the relation between structure and function of the breast provides ample evidence of the frequency with which hindrance to outflow is caused by anatomical factors.

SUMMARY

Data are presented showing the level of, and changes in, viscosity, total nitrogen, globulin and casein in human mammary secretions during late pregnancy and early lactation. In addition a gold sol reaction is described which permits the rapid determination of a factor, the 'gold number', shown to be linearly connected with the total nitrogen content of the secretions.

The results show that regular removal of the antenatal fluid does not lead to systematic alterations in composition. Characteristic changes of the factors investigated occur during the first few days *post partum*, and it is proposed to confine the use of the term 'colostrum' to the fluid secreted during this period.

The data are interpreted as suggesting faults in the mechanism of expulsion rather than faults in the mechanism of milk production as the principal cause of the lactational difficulties encountered so frequently during the early postnatal stages.

The authors are indebted to Prof. H. D. Kay, Dr G. W. Scott Blair and Dr S. J. Rowland for their continued interest and much helpful advice, and to Dr C. A. Mawson for arranging the supply of specimens from patients of the Royal Berkshire Hospital.

REFERENCES

Asdell (1925). *J. agric. Sci.* **15**, 358.
Bell (1928). *J. biol. Chem.* **80**, 239.
Einstein (1906). *Ann. Phys., Paris*, **19**, 289.
Erickson, Gulick, Hunscher & Macy (1934). *J. biol. Chem.* **106**, 145.
Lowenfeld, Widdows, Bond & Taylor (1927). *Biochem. J.* **21**, 1.
—— & Widdows (1928). *J. Obstet. Gynaec.* **35**, 114.
Pennycuick, Woolcock & Cowan (1938). *J. Path. Bact.* **46**, 549.
Rowland (1938). *J. Dairy Res.* **9**, 30, 42.
Scott Blair (1941). *Biochem. J.* **35**, 267.
—— Watts & Denham (1927). *Cereal Chem.* **4**, 63.
Seibert (1926). *J. biol. Chem.* **70**, 265.
Seitz (1924–25). *Arch. Gynaek.* **123**, 46.
Waller (1938). Clinical Studies in Lactation. London: William Heinemann, Ltd.
Widdows, Lowenfeld, Bond & Taylor (1930). *Biochem. J.* **24**, 327.
—— —— —— Shiskin & Taylor (1935). *Biochem. J.* **29**, 1145.
Woodman & Hammond (1922). *J. agric. Sci.* **12**, 97.
—— —— (1923). *J. agric. Sci.* **13**, 180.

29. THE COLORIMETRIC ESTIMATION OF TOTAL CHOLESTEROL IN WHOLE BLOOD, SERUM, PLASMA AND OTHER BIOLOGICAL MATERIAL

BY J. T. IRELAND

From the Research Institute for the Prevention of Disease, Liverpool, and Liverpool Heart Hospital

(*Received 7 February 1941*)

SINCE Autenrienth & Funk [1913] and Weston [1917] first drew attention to its inaccuracies, the colorimetric method for the estimation of cholesterol based on the Liebermann-Burchard reaction [Grigaut, 1910] has undergone many modifications. It has been the author's experience, however, that no method yet published has succeeded in entirely overcoming the difficulties mentioned by these authors.

With the majority of modern photoelectric colorimeters the colour produced in the test solution alone is measured and the required result is obtained by consulting a previously drawn calibration chart, not by comparing the colour with that of a standard simultaneously developed. It is essential therefore that the colour be exactly reproducible. With this in mind a study was made of existing methods of colour development [Bloor, 1913–14; 1914; 1915; 1916; 1928; Autenrienth & Funk, 1913; Cornell, 1928; Schoenheimer & Sperry, 1934; Reinhold & Sheils, 1936], with a view to adapting them for use with the photoelectric colorimeter. Using a standard solution of cholesterol in chloroform, not one of the above methods gave satisfactorily reproducible results, and furthermore, when the standard solution was replaced by a cholesterol solution prepared from blood by existing methods of extraction [Bloor, 1913–14, Sackett, 1925; Myers & Wardell, 1918; Reinhold & Sheils, 1936], the variations were accentuated in all cases.

The object of this investigation was therefore to examine the factors influencing the accuracy of the colorimetric method for the estimation of cholesterol and to utilize the results obtained to devise a more accurate method.

The investigation fell into two sections: the study of (*a*) methods of extraction of cholesterol from blood; (*b*) methods for the development of the colour.

Extraction methods. Two general methods exist for the extraction of cholesterol: (1) chloroform extraction of blood dried by heat or by dehydrating agents [Myers & Wardell, 1918; De Toni, 1926; Leiboff, 1924; Ling, 1928; Reinhold & Sheils, 1936]; (2) extraction from fresh blood by means of alcohol, alcohol-ether, acetone etc. These latter methods are generally accepted as being more complete, and that due to Bloor [1913–14] is the one most frequently used. Their great disadvantage lies in the fact that the extract has a yellow brown colour which leads to high and erratic results [Mueller, 1916; Weston, 1917; Luden, 1917]. Various methods have been suggested for the elimination of this interference. Mueller [1916] washed the chloroform extract with water and then dried it with anhydrous sodium sulphate. Stoddart & Drury [1929] and others have advocated saponification before solution in chloroform. Sackett [1925] claimed that the colour could be avoided if the ether-alcohol extraction was carried out without heating. Cantoni [1930] suggested adding a mixture of oleic and palmitic acids to the standard to compensate for the colour. Bloor [1928] advised the use of a red glass filter in the colorimeter when there was any serious degree of discoloration. Schoenheimer & Sperry [1934] and Kelsey [1939] avoid this difficulty by saponifying the extract and removing the cholesterol as digitonide, which is then purified and analysed.

Methods for the development of the colour. The following are the conditions laid down in existing methods for the control of the development of the green colour produced by the interaction of acetic anhydride and sulphuric acid with cholesterol in chloroform solution:

Bloor [1913–14; 1928]. Stand for 15 min. in the dark or in the light in which the comparison is to be made—no temperature given.

Autenrienth & Funk [1913]. 15 min. at 35°.

Weston [1917]. Add H_2SO_4, mix, stand in dark 30 min., then add the acetic anhydride and stand 15 min.

Cornell [1928]. 15 min. away from light at laboratory temperature.

Schoenheimer & Sperry [1934]. Warm solution to 25° before adding reagents, develop colour in water bath at 25° and read between 27–37 min. The amount of H_2SO_4 added is described as 0·1 ml. (or 4 uniform drops).

Reinhold & Sheils [1936]. 30 min. at 25° in the same light in which the comparison is to be made. The solution is not previously heated but warm acetic anhydride-sulphuric acid mixture is added.

Kelsey [1939]. Set in water bath at 23° for 15 min. under moderate conditions of lighting.

The factors concerned are therefore: light, concentration of reagents and of cholesterol itself, temperature and time, and one other not mentioned above, namely, the condition of the cholesterol whether free or esterified. Some of the above methods use extracts prepared directly from blood and therefore containing both esterified and free cholesterol, others use saponified extracts containing only free cholesterol. Gardner & Williams [1921] found that esterified cholesterol reacted at a greater rate than free cholesterol and that cholesteryl acetate gave an 'apparent cholesterol' 10 % greater than an equivalent amount of free cholesterol; cholesteryl benzoate gave values 4·6 % greater than free cholesterol. Kelsey [1939] found that the colour produced by cholesteryl palmitate was 130 % of that produced by an equivalent amount of free cholesterol. Reinhold & Sheils [1936], using their method of colour development, confirmed the findings of Gardner & Williams [1921] and reported an 'apparent cholesterol' for cholesteryl palmitate of 120 %. Yasuda [1936] has reported the palmitate as giving 24·5 %, the stearate 25·2 % and the oleate 27·6 % greater values than an equivalent amount of free cholesterol.

EXPERIMENTAL

The factors controlling the development of the colour were studied first in order to try to evolve a standard method which could be used to compare the various extraction methods. Each factor was investigated in turn while the others were kept as constant as possible.

Light. It was found that light had a marked effect on the colour development, as will be seen in the following experiment, in which two series of exactly similar solutions of cholesterol were treated alike in every respect except that the colour development in one series took place in the dark and in the other in the light.

	Colour developed in light Absorption density 640 mμ	Colour developed in dark Absorption density 640 mμ
1	0·357	0·387
2	0·362	0·387
3	0·355	0·387
4	0·333	0·389
5	0·352	0·382

The series developed in the light, it will be seen, gave paler solutions which varied in intensity, due probably to unequal lighting. Hence in all subsequent experiments the colour was allowed to develop in the dark.

Concentration of reagents. Alteration of the concentration of the reagents, particularly that of H_2SO_4, gave rise to changes in the final colour.

	ml.	Absorption density 640 mμ
Solution A	4·7 standard cholesterol solution 0·2 chloroform 0·1 conc. H_2SO_4 2·0 acetic anhydride	0·387
Solution B	4·7 standard cholesterol solution 0·1 chloroform 0·2 conc. H_2SO_4 2·0 acetic anhydride	0·373
Solution C	4·7 standard cholesterol solution 0·3 conc. H_2SO_4 2·0 acetic anhydride	0·367

This difficulty can only be overcome by standardizing the amounts of reagents present. In this investigation 2 ml. of a mixture, prepared not more than 10 min. previously, of acetic anhydride A.R. (20 ml.) and conc. H_2SO_4 A.R. (1 ml.) were added to every 5 ml. of chloroform solution of cholesterol.

The concentration of cholesterol itself is another factor to be considered. Methods quoted above use concentrations varying from 3·0 to 0·2 mg. per 5 ml. chloroform (i.e. amounts roughly equivalent to those present in 1·5–0·1 ml. of normal serum). Concentrations of 0·2–0·6 mg. per 5 ml. were taken in these experiments, largely because the depth of colour produced was convenient for the photoelectric colorimeter used. Later experiments showed that with higher concentrations of cholesterol the colour formation tended to become erratic.

The two remaining factors temperature and time were considered together. Fig. 1 shows the relation between absorption density and time at 18°, 25° and 35°, all other factors being standardized.

Each point was determined under the following conditions. To 5 ml. of a standard solution (=0·289 mg.) of cholesterol in chloroform at 18°, 25° or 35° were added 2 ml. of a mixture of acetic anhydride and conc. H_2SO_4 (20 : 1) freshly prepared and warmed to the same temperature. After careful mixing the solution was placed in a beaker of water maintained at the required temperature in the dark and removed at the expiration of the required time. The same tubes were used throughout, being as far as possible of the same thickness, to prevent irregularities of heating. The readings were taken on a Bolton-Williams type of photoelectric colorimeter, using as the blank 5 ml. of chloroform with 2 ml. of acetic anhydride-sulphuric acid mixture which had been treated in the same way as in the test. The absorption of light (640 mμ) by a layer of solution 1 cm. in thickness was measured and the result was converted into absorption density (which is proportional to cholesterol content).

It will be seen from the curve that the colour is partially destroyed by heat, the maximum point attained decreasing with temperature. The curve for 35° shows that this temperature is obviously unsuitable for colorimetric purposes, the changes of colour being too fast to give adequate time for a reading to be made.

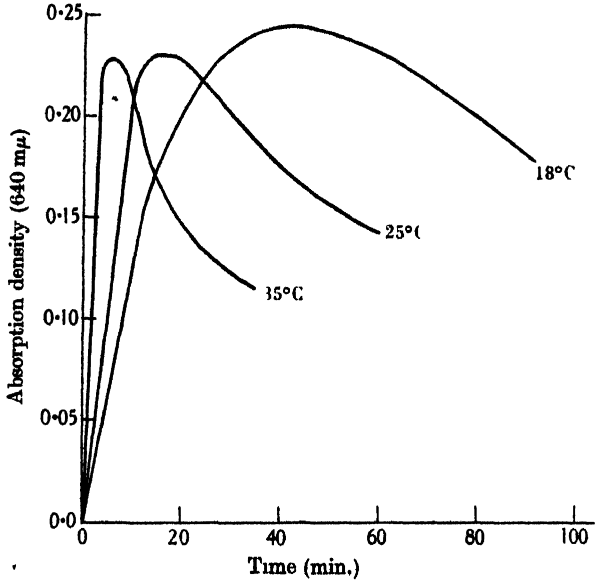


Fig. 1. Colour development charts for free cholesterol at 18°, 25° and 35°.

The same may be said with less force of the 25° curve, the rate of change corresponding to as much as 2·1 mg./100 ml. in the original blood per min. at 30 min.

Further, 25° is a difficult temperature to maintain exactly in a small laboratory without special apparatus for the purpose. Small differences in heating due to outside factors such as differences in the thickness of the tubes cause appreciable variations. At 25° and 30 min. twenty-five estimations gave: average absorption density 0·209 (145) ± 0·015; max. 0·223 (155) and min. 0·190 (131). (The figures in brackets give the approximate equivalent in terms of mg./100 ml. normal serum.)

The optimum temperature proved to be 18° and the time 60 min., the fading of the colour being sufficiently slow to permit an accurate reading to be made, even when a number of estimations are being carried out at the same time. It was also quite easy to maintain this temperature. Results, using these conditions, were exactly reproducible. Twenty-five estimations on the same solution gave an average absorption density 0·234 (145) ± 0·002 with max. 0·236 (147) and min. 0·232 (143).

Another factor not yet mentioned is the presence of water in the reagents, particularly the chloroform. Previous methods have insisted on the use of water-free reagents without giving any explanation for this precaution. This is really only another demonstration of the influence of temperature on the reaction. The heat evolved by the addition of conc. H_2SO_4 to chloroform containing only traces of water is sufficient to accelerate the colour development and the subsequent fading and hence lower the final result. All reagents should therefore be completely water-free.

Condition of the cholesterol. The last factor, namely, the state of the cholesterol, was investigated as follows. In each of four 100 ml. conical flasks were placed 30 ml. of chloroform containing 1·794 mg. of cholesterol as (1) free cholesterol, (2) cholesteryl acetate, (3) cholesteryl oleate, (4) cholesteryl stearate. The four flasks were then placed in the dark in a water bath maintained at 18°. To each were added 12 ml. of a freshly prepared mixture of acetic anhydride and conc. H_2SO_4 cooled to 18°. The absorption of light of wave-length 640 mμ by each of the solutions was measured at frequent intervals and a chart prepared showing the relation between time and absorption density (colour) of the solutions (Fig. 2).

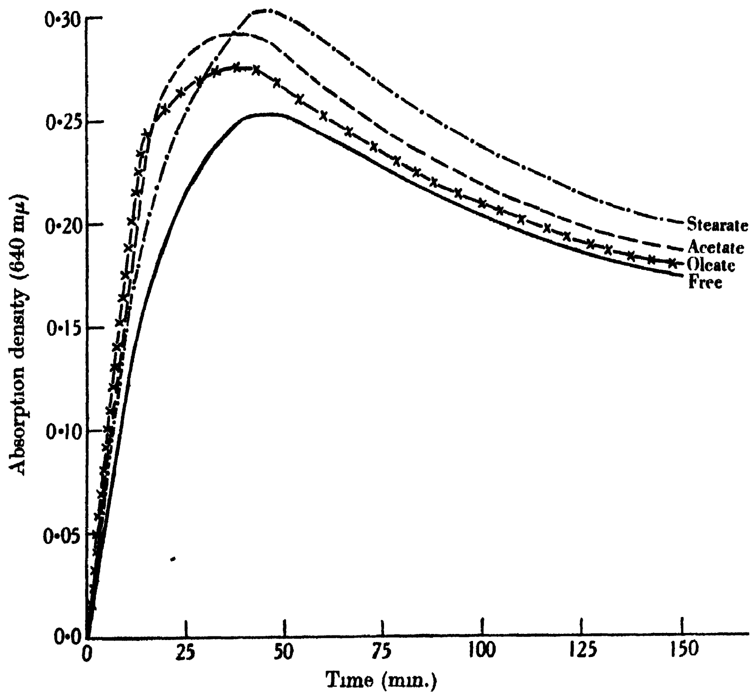


Fig. 2. Colour development charts for cholesterol and its esters (18°). Key: Cholesterol ——. Cholesteryl oleate ×—×. Cholesteryl acetate — —. Cholesteryl stearate —·—.

The following points are worthy of note.

(1) The four curves are similar in shape.

(2) All esters show a faster colour development, a deeper maximum colour, and a faster colour fading than free cholesterol.

(3) The difference between cholesterol and its esters is greatest in the region of maximum colour development and diminishes as the colours fade, tending to become constant; readings taken after 24 hr. still showed higher values for esters than free cholesterol.

(4) Each ester has its own characteristic rate of colour development and also maximum colour. After a certain time the rate of colour fading is approximately the same in all cases.

(5) This experiment was repeated at various temperatures, but no conditions could be found in which the same amount of cholesterol gave an identical colour in all four forms.

The above facts explain the great diversity in the 'apparent' cholesterol values obtained by previous workers for the various esters. Using the conditions specified above, the 'apparent' cholesterol values for cholesteryl stearate, oleate and acetate are respectively 119, 103·2 and 109·2 % of that for free cholesterol.

The obvious method of eliminating this difference between the various forms is to convert them into one common form; saponification presents the easiest way of doing this. Difficulties were encountered in adapting existing methods. To test them, two chloroform solutions were prepared, each containing the same weight of cholesterol per unit volume, one as free cholesterol, the other as cholesteryl stearate. 5 ml. portions of the cholesteryl stearate solution were taken and after removal of chloroform subjected to the various procedures suggested for the saponification of blood fats; they were then redissolved in 5 ml. chloroform. The 'apparent' cholesterol content of the saponified extracts and also that of 5 ml. of the original cholesteryl stearate solution were determined colorimetrically (using the procedure developed above) by comparison with the colour simultaneously developed by 5 ml. free cholesterol solution.

The following are some typical results taken at random from a large number of analyses to illustrate the type of results obtained.

		'Apparent' cholesterol mg.		
Material	Method of saponification	(*a*)	(*b*)	Remarks
0·386 mg. free cholesterol	None	0·386	0·386	Blank
0·652 mg. cholesteryl stearate	None	0·463	0·463	Actual cholesteryl content = 0·386 mg.
0·386 mg. free cholesterol	Aqueous KOH (10 %)	0·386	0·386	Test of extraction by chloroform (*v. infra*)
,,	,,	0·384	0·386	
0·653 mg. cholesteryl stearate	Aqueous KOH (10 %), 3 hr. in sealed flask	0·415	0·422	Chloroform extraction. Extract colourless
0·652 mg. cholesteryl stearate	1·0 *N* sodium ethylate [Bloor, 1928]	0·360	0·370	Light petroleum extraction. Extract yellow
,,	,,	0·410	0·418	Chloroform extraction. Extract yellow
,,	Alcoholic (70 %) KOH (10 %), 3 hr. at 40°, alcohol evaporated off at 70°	0·431	0·423	Chloroform extraction. Extract yellow
,,	Alcohol-acetone KOH (10 %), 3 hr. at 40°, alcohol-acetone evaporated off at 70°	0·425	0·440	Chloroform extraction. Extract yellow
,,	2 ml. aqueous KOH (10 %), 1 ml. amyl alcohol, 90 min. at 100° in stoppered flask	0·386	0·386	Chloroform extraction. Extract colourless

A serious difficulty encountered here was the fact that in the majority of the methods, after saponification the cholesterol is not extracted from the aqueous-alcohol media as such but is precipitated as cholesteryl digitonide. Early experiments after the method of Bloor [1928], using light petroleum as solvent, gave rather low results (see table). Light petroleum, while being a good general fat solvent, is not a sufficiently good one for cholesterol. Attempts were therefore made to extract cholesterol quantitatively from strong aqueous alkaline solutions by means of chloroform, which is easily the best cholesterol solvent. As will be seen from the table, 100 % recovery was obtained of cholesterol added to 2 ml. portions of a 10 % aqueous solution of KOH. Details of the actual technique used are given later in the section on method.

The general conclusion to be drawn from the above table is that alcohol is unsuitable as a solvent during saponification for colorimetric analysis, since, wherever it is used, the final chloroform extract is tinted yellow, and since this colour is increased by heat it is difficult to apply sufficient heat to the system to keep the cholesterol in solution and to obtain complete saponification. Amyl alcohol, on the other hand, although less miscible with water has the advantage that it is less volatile and no colour is produced during saponification. 2 ml. of 10% aqueous KOH and 1 ml. of amyl alcohol at 100° in a closed flask for 90 min. completely saponified 0·652 mg. of cholesterol stearate. Complete saponification of cholesteryl acetate and oleate was also obtained by this same method. The application of the method to cholesterol-containing extracts prepared from blood will be considered after the next section.

Extraction methods. Only the standardized method of colour development evolved above was used in the analysis of the various extracts referred to in this section.

All methods involving the drying of blood gave consistently low results and poor recovery of added cholesterol. It was found difficult, if not impossible, to obtain complete extraction of cholesterol under these conditions, a conclusion agreeing with that of other workers in this field.

As regards 'wet' methods, the yellow colour of the extract already referred to presents a big difficulty. Below are some of the results obtained in experiments carried out to test the suggestions quoted earlier for removing it.

The colours of chloroform extracts prepared from the same serum by the various methods were compared in terms of their absorption of light of wavelength 440 mμ.

		Percentage absorption of light, 440 mμ	
Extraction method	Method of purification	(*a*)	(*b*)
Hot ether-alcohol	None	9·0	9·5
Hot ether-alcohol	Mueller's method	9·0	9·0
Hot ether-alcohol	Saponification (sodium ethylate)	11·0	12·5
Cold ether-alcohol	None	7·0	6·5

The remaining methods were found to be equally ineffective in overcoming the difficulty. It will be noted that results from Sackett's method indicate that heating in Bloor's original method increases the colour, as also does saponification with sodium ethylate.

Theoretically the procedure advocated by Schoenheimer & Sperry [1934] and Kelsey [1939], viz. saponification of extract, removal of cholesterol as digitonide, its saponification and finally its analysis, is more accurate, but it is open to criticism from a number of points of view. For clinical purposes it is too long and complicated and the number of stages involved tends to increase the possibility of error. In practice it was found difficult to isolate quantitatively so small an amount of the very light particles of cholesteryl digitonide merely by centrifuging them. The method of colour development is also open to criticism; in addition to the points already noted, i.e. time, temperature, concentration of reagents etc., it was noted that the digitonin present in the solution after the liberation of the cholesterol from the digitonide itself gave a reddish-purple colour with the acetic anhydride-sulphuric acid mixture. To compensate for this Schoenheimer & Sperry advocated the use of colour filters. My experiments on these lines indicated that whatever colour screen was used some light absorption could always be detected.

It was therefore decided to revert to Bloor's original ether-alcohol extract and endeavour to find some method whereby it could be decolorized without loss of cholesterol. This was finally accomplished as follows. When a yellow-brown chloroform extract prepared from blood by Bloor's method was passed through a column of finely divided silica gel, the issuing fluid was found to be completely colourless. When solutions of pure cholesterol were passed through, followed by chloroform washings, 100 % recovery of cholesterol was obtained in every case.

The following are some typical results.

	Optical colorimeter			Photoelectric colorimeter		
	A	B	C	A	B	C
Bloor's extract untreated, coloured	205	196	220	194	190	216
Ditto after silica treatment, colourless	190	187	210	190	188	212
Ditto plus added cholesterol equivalent to 50 mg./100 ml. after silica treatment, colourless	240	237	260	240	238	262

As would be expected results are lower after the removal of the interfering colour. The fact that the difference is less marked in the case of the photoelectric colorimeter can be accounted for as follows. The green colour was measured in terms of its percentage absorption of light of wave-length 640 mμ (red brown); the percentage absorption of this wave-length by the similarly coloured solutions before colour development averaged only 0·8 %, which is equivalent to an apparent cholesterol of 4 mg./100 ml. original serum. This, it will be seen, corresponds with the average difference between the results before and after silica treatment. The brown pigment is apparently not affected by the sulphuric acid-acetic anhydride mixture [cf. Luden, 1917] and therefore in cases where extreme accuracy is not required it is justifiable, in the case of serum or plasma, to omit the silica treatment provided that the colour is measured in terms of its percentage absorption or transmission of light of wave-length 640 mμ. When estimating the cholesterol content of whole blood or red blood cells the interfering colour is much stronger and should always be removed.

The practical details of the silica treatment are given later in the section on method. In addition to blood the method has proved of even greater value in tissue analysis, since the brown colour which is often present in larger amounts in tissue extracts is completely removed by the silica.

Application of saponification method to blood extracts. The cholesterol content of a number of 2 ml. samples of whole blood and serum was determined by the following procedure: extracting with ether-alcohol, filtering, evaporating off the ether-alcohol, extracting the residue with chloroform, decolorizing the chloroform extract, removing the chloroform, saponifying and finally extracting the cholesterol for colorimetric analysis, according to the conditions already noted. Simultaneously, further ether-alcohol extracts of 2 ml. samples of the same blood and serum were prepared and to these were added known amounts of cholesterol or cholesterol ester. The total cholesterol content was then measured in the same manner as above. The results (below) showed a good recovery of added cholesterol, indicating that the figures for whole blood and serum were also consistent.

	Serum A, mg. cholesterol		Serum B, mg. cholesterol		Blood C, mg. cholesterol		Blood D, mg. cholesterol	
Material	1	2	1	2	1	2	1	2
0·2 ml. blood or serum	0·305	—	0·352	—	0·210	—	0·242	—
0·2 ml. blood or serum + 0·193 mg. free cholesterol	0·500	0·195	0·545	0·193	0·404	0·194	0·435	0·193
0·2 ml. blood or serum + 0·193 mg. cholesterol as stearate	0·498	0·193	0·545	0·193	0·405	0·195	0·436	0·193
0·2 ml. blood or serum + 0·193 mg. cholesterol as oleate	0·498	0·193	0·545	0·193	0·403	0·193	0·432	0·190

The figures in the second columns give the actual cholesterol recovery.

METHOD

Preparation of ether-alcohol extract

2 ml. of whole blood, serum or plasma are run slowly into 60 ml. of freshly mixed ether-alcohol (3/2) in a 100 ml. conical flask, the liquid being gently shaken with a swirling motion. The flask is then immersed in hot (80°) water and rotated continuously to prevent superheating. When the contents of the flask have been brought to the boil it is removed and cooled in running water to room temperature. The solution is next filtered into a dry graduated 100 ml. flask, the conical flask and the residue on the paper being washed several times with further quantities of warm ether-alcohol mixture. When cool the combined filtrates are made up to 100 ml. and well mixed.

Removal of colour

50 ml. of this solution are transferred to a 100 ml. squat-form beaker and evaporated just to dryness on a water bath. The residue is then extracted three times, twice with 5 ml. and once with 3 ml. of warm dry chloroform, the extracts being decanted into a small clean dry separating funnel.

A silica adsorption tube is meanwhile prepared as follows. A clean dry test tube $6 \times \frac{1}{2}$ in. is heated in the bunsen flame about 1 in. from the sealed end and drawn out to a capillary (about $\frac{1}{12}$ in. bore) which is cut 1 in. from the main part of the tube. When cool, a lightly packed plug of cotton wool is placed in the tube at the point where it begins to narrow, and the tube is filled to within $\frac{3}{4}$ in. of the top with finely divided pure silica (see below). A second plug of cotton wool is placed on top of the silica. The tube is fitted by means of a rubber stopper into the neck of a side-arm test tube (30 ml. capacity), or small filter flask, which is connected to the filter pump. 5 ml. of dry chloroform are allowed to flow through the adsorption tube and the residue is removed by suction. The receiver is changed and first the chloroform extract and then 15 ml. of pure dry chloroform are allowed to run through (without suction) at the rate of 15–20 drops per min.; the final portion of the chloroform is removed from the silica by turning on the filter pump, slowly at first, then at full pressure, for 5 min. The chloroform extract and washings are completely transferred to a dry 50 ml. graduated flask and made up to volume; the flask is stoppered and well shaken to ensure thorough mixing.

Saponification of esters

10 ml. of chloroform solution are pipetted into a clean dry 120 ml. pyrex conical flask fitted with a ground glass stopper. The flask, unstoppered, is placed in a steamer (if a bacteriological one is not available one of the household kind can be made to serve the purpose), until all the chloroform has been removed. 1 ml. of amyl alcohol is then pipetted into the flask, followed by 2 ml. of 10% aqueous KOH. After its contents have been well mixed by shaking, the flask is replaced in the steamer. Twenty minutes later the stopper is placed in position and the heating continued for 90 min.: from time to time the flask, without being removed from the steamer, is given a swirling motion to agitate its contents. (Care should be taken that the stopper is not blown out.) At the end of the required time the flask is removed from the steamer and the stopper withdrawn while still hot. When the contents of the flask have cooled to 40°, 5 ml. of chloroform are run in and the flask is then shaken with a vigorous swirling motion, care being taken not to spill any of the contents. After allowing a few

minutes for it to settle, the chloroform-water mixture is decanted into a clean dry test tube (thick walled, 30 ml. capacity), the last few drops being washed in with the aid of a chloroform wash bottle. The stoppered flask is then carefully washed out with two 3 ml. portions of warm chloroform, the washings being added to the main chloroform-water mixture in the tube. The test tube is filled to within $\frac{3}{4}$ in. from the top of the tube with distilled water, tightly stoppered and inverted several times. When the two layers have separated, the tube is given a number of sharp taps at about the level of the water meniscus to dislodge the small drops of chloroform that usually remain in the meniscus, and it is then replaced in the rack for a further 3 min. Having made sure that all the chloroform has settled into the lower layer, the bulk of the upper layer is drawn off by means of gentle suction, using a narrow glass tube drawn to a jet, the tip of which is turned upwards. An aqueous layer about $\frac{1}{8}$ in. deep is left above the chloroform and to this are added 10–12 ml. of N HCl; the tube is then stoppered and inverted several times and the contents are allowed to settle as before. The HCl washing is repeated twice, followed by three water washings in the same manner. After the final water washings have been removed the chloroform extract is decanted into a clean dry 50 ml. beaker and the tube is washed out twice with 2 ml. portions of chloroform, the washings being added to the main bulk of the solution. The beaker is then placed on a hot water bath at 80° and the extract evaporated just to dryness. The residue is dissolved by warming with 2 ml. of pure dry chloroform, the solution is decanted with the aid of a glass rod and chloroform wash bottle into a small test tube ($5 \times \frac{1}{2}$ in.) graduated at the 5 ml. mark. The beaker is washed out with further portions of chloroform and when cold the combined extracts are made up to 5 ml. with dry chloroform.

Development of colour

The tube is corked and placed in a water bath maintained at 18° and side by side with it is placed a test tube containing a freshly prepared mixture of 20 : 1 acetic anhydride and H_2SO_4 (both A.R.). After 10 min. the two tubes are withdrawn and 2 ml. of the acetic anhydride-sulphuric acid mixture are pipetted into the extract. The contents of the tube are well mixed and the tube is placed in the water bath at 18° in the dark. After 60 min. the tube is removed and its percentage absorption of light of wave-length 640 mμ is measured in the Bolton-Williams type of photoelectric colorimeter, using a blank consisting of 5 ml. of chloroform and 2 ml. of acetic anhydride-sulphuric acid mixture. The quantity of cholesterol per 100 ml. of blood or serum can then be read off from calibration charts previously prepared, using standard quantities of cholesterol under the same conditions.

For photometric measurements, using the Pulfrich photometer, one cell is filled with the blank solution and the unknown is pipetted into the other. The percentage transmission (T) is read (see manufacturer's directions).

Observed $=(-\log(T/100))/L$, where $L=$ length of cell.
$E_{\text{sp.}}=$ specific extinction coefficient of cholesterol.
Then $E/E_{\text{sp.}}=$ mg. cholesterol in sample.

The amount of cholesterol per 100 ml. blood = 500 times the amount in sample.

For the ordinary optical colorimeter, standards containing 0·5, 0·4 and 0·3 mg. cholesterol per 5 ml. are prepared and the colour is developed simultaneously with the test solutions. The test is compared with the standards and the results are obtained by the usual S/T ratios.

Preparation of silica for adsorption tube

The more obviously iron-containing particles are removed by hand from ordinary silica gel as purchased. The remaining particles are crushed with the aid of a mortar and pestle and are then transferred to a large flat-bottomed pyrex flask and more than covered with H_2SO_4 (1 vol. acid to 2 vol. water). The flask is next heated with constant shaking for about 20 min. in the fume cupboard. When cold the H_2SO_4 is decanted off and the silica washed several times with warm water; for the final washing the silica is left standing in contact with the water overnight. The purified silica is dried in a steam-heated oven for several hours. Only those particles of the pure silica which pass through a mesh $\frac{1}{20}$ in. diameter and yet not through one $\frac{1}{32}$ in. are used for the adsorption tube.

SUMMARY

1. It has been demonstrated that existing methods for the development of the green colour by the interaction of acetic anhydride and sulphuric acid with cholesterol in chloroform solution do not sufficiently take into account all the factors concerned. These factors have been studied, and from the results obtained an improved method of colour development has been suggested.

2. It has also been demonstrated that the yellow-brown colour in ether-alcohol extracts from biological material, which has been so frequently referred to, can be removed without loss of cholesterol.

3. On the basis of these facts an improved method for the colorimetric estimation of cholesterol in biological material has been described.

I am extremely indebted to Dr I. Harris for his valuable assistance and advice in the preparation of this paper, and to the Lord Leverhulme Trust Fund whose financial assistance made it possible.

REFERENCES

Autenrienth & Funk (1913). *Münch. med. Wschr.* **60**, 1243.
Bloor (1913–14). *J. biol. Chem.* **16**, 517.
—— (1914). *J. biol. Chem.* **17**, 377.
—— (1915). *J. biol. Chem.* **23**, 317.
—— (1916). *J. biol. Chem.* **27**, 106.
—— (1928). *J. biol. Chem.* **77**, 53.
Cantoni (1930). *Diagn. Tec. Labo., Napoli,* **1**, 535.
Cornell (1928). *J. Lab. clin. Med.* **14**, 251.
De Toni (1926). *J. biol. Chem.* **70**, 207.
Gardner & Williams (1921). *Biochem. J.* **15**, 371.
Grigaut (1910). *C.R. Soc. Biol., Paris,* **118**, 827.
Kelsey (1939). *J. biol. Chem.* **127**, 15.
Leiboff (1924). *J. biol. Chem.* **61**, 177.
Ling (1928). *J. biol. Chem.* **76**, 361.
Luden (1917). *J. biol. Chem.* **29**, 463.
Mueller (1916). *J. biol. Chem.* **25**, 549.
Myers & Wardell (1918). *J. biol. Chem.* **36**, 147.
Reinhold & Sheils (1936). *Amer. J. clin. Path.* **6**, 22.
Sackett (1925). *J. biol. Chem.* **44**, 203.
Schoenheimer & Sperry (1934). *J. biol. Chem.* **106**, 745.
Stoddart & Drury (1929). *J. biol. Chem.* **84**, 741.
Weston (1917). *J. biol. Chem.* **28**, 383.
Yasuda (1936). *J. Biochem., Tokyo,* **24**, 429.

30. SOME APPLICATIONS OF PERIODIC ACID TO THE STUDY OF THE HYDROXYAMINO-ACIDS OF PROTEIN HYDROLYSATES

1. THE LIBERATION OF ACETALDEHYDE AND HIGHER ALDEHYDES BY PERIODIC ACID

2. DETECTION AND ISOLATION OF FORMALDEHYDE LIBERATED BY PERIODIC ACID

3. AMMONIA SPLIT FROM HYDROXYAMINO-ACIDS BY PERIODIC ACID

4. THE HYDROXYAMINO-ACID FRACTION OF WOOL

5. 'HYDROXYLYSINE'

By A. J. P. MARTIN and R. L. M. SYNGE[1]

Wool Industries Research Association, Torridon, Headingley, Leeds

With an Appendix by

FLORENCE O. BELL

Textile Physics Laboratory, University of Leeds

(*Received 25 February 1941*)

Largely because of the lack of satisfactory analytical procedures, the hydroxyamino-acids are at present the least well investigated category of protein constituents, in spite of the fact that the simplest hydroxyamino-acid, serine, was one of the earlier amino-acids to be discovered in proteins [Cramer, 1865].

Nicolet & Shinn [1939] showed that periodic acid, a reagent introduced into organic chemistry by Malaprade [1934] for the splitting of α-glycols, also splits serine, the products of oxidation being one molecule of formaldehyde and, probably, one molecule each of ammonia and glyoxylic acid. Van Slyke *et al.* [1940] have subsequently used this reagent for the study of the new base 'hydroxylysine', and have mentioned a simple method for the detection of amino-acids having an —OH and an —NH_2 group in adjacent positions, by the liberation of NH_3 in the presence of periodate in strong K_2CO_3 solution.

Block & Bolling [1939] have made use of lead tetra-acetate, which reacts with α-glycols in the same way as periodic acid [Criegee *et al.* 1933], to determine threonine in protein hydrolysates. These workers determined the acetaldehyde liberated by a specific colorimetric method.

In the present work we have studied the reaction between periodic acid and hydroxyamino-acids both in solutions of known composition and in preparations derived from proteins.

---

[1] International Wool Secretariat Student.

This has led to the development of analytical procedures which, though quantitatively not all that could be desired, are of high specificity, rapid, unlaborious and require only small amounts of material. They promise, therefore, to be of use in the study of proteins and preparations derived therefrom both for the analysis of complete hydrolysates and for the control of isolative procedures.

I. THE LIBERATION OF ACETALDEHYDE AND HIGHER ALDEHYDES BY PERIODIC ACID

[Cf. Martin & Synge, 1940]

On treatment with periodic acid, threonine yields acetaldehyde, and this may be separated from the formaldehyde resulting from serine, 'hydroxylysine' etc. by simple aeration, since formaldehyde, unlike its higher homologues, is not significantly volatile from aqueous solution [cf. Blair & Ledbury, 1925; Ledbury & Blair, 1925; Walker, 1940].

So far as we are aware, this difference between formaldehyde and its homologues has not previously been made use of for analytical purposes.

EXPERIMENTAL

In the present work, acetaldehyde was aerated from the reaction mixture in a 50 ml. washing tube directly into an absorption tower (separating funnel packed with glass beads and mounted in a Buchner flask, as shown in Fig. 1) containing 25 ml. of 0·3 % aqueous $NaHSO_3$. After rapid aeration for 30 min. the solution and washings from the tower were titrated in the Buchner flask with $N/300$ iodine solution [Friedemann & Kendall, 1929].

By placing a second absorption tower in series with the first, it was found that 95 % absorption of acetaldehyde took place in the first tower. The figures for volatile aldehyde given subsequently in this paper are not corrected for this and refer to aldehyde as titrated in the single absorption tower.

On aeration of 5·35 μmol. of acetaldehyde from 25 ml. of water in the aeration vessel, recoveries of 87–92 % were obtained, and these were not affected by the presence of 8·75 μmol. of formaldehyde in the acetaldehyde solution. Blank runs showed that the laboratory air was substantially free from aldehyde, and in no case was a 'reagent blank' found.

Yield of acetaldehyde from threonine

In all these tests synthetic *dl*-threonine was used. *dl-allo*-Threonine, prepared from *O*-methyl-*dl-allo*-threonine [cf. West & Carter, 1937], gave an identical yield, while samples of threonine of natural origin prepared from rabbit myosin by Sharp [1939] gave 70–72 % of the yield given by the synthetic material.

Amounts of 0·5–1·5 mg. of threonine were treated at room temperature with 50 mg. of periodic acid (B.D.H.) in 25 ml. total volume of aqueous solution. The yield of acetaldehyde obtained in the absorption tower after 30 min. rapid aeration was found to be dependent on *p*H, as shown in Table 1.

A definite optimum yield at about *p*H 7 is to be noted. The reasons for the lower yields on either side of this optimum, and for the incomplete yield at the optimum, have not been investigated in detail; in acid solution neither periodic acid, iodic acid nor ammonia interferes with the removal of added acetaldehyde

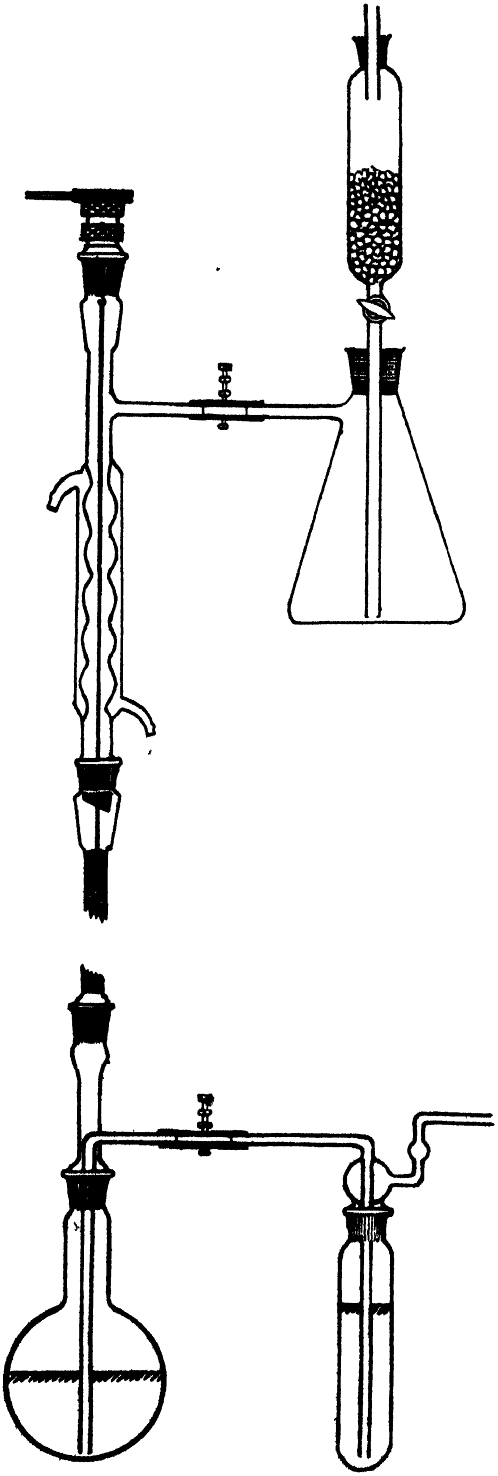


Fig. 1. Apparatus for micro-separation of aldehydes.

Table 1. *Yield of acetaldehyde from* dl-*threonine treated with periodic acid in various solutions*

Solutions used	Yield of acetaldehyde %
Water	9–10
$M/5$ acetate buffer, pH 4·7	52
$M/5$ $NaHCO_3$	66–72
$M/5$ phosphate buffer, pH 7·2	69
'Old' $M/5$ $NaHCO_3$, become more alkaline by loss of CO_2	55
50 % w/v K_2CO_3 solution	45

from aqueous solution. It is therefore probable that, in acid solution, threonine is either only slowly attacked by periodic acid or that side reactions result in products other than acetaldehyde. On the alkaline side it seems possible that the simultaneously liberated ammonia interferes with the determination, or that acetaldehyde is slowly oxidized by the reagents.

All determinations were accordingly carried out in fresh $M/5$ $NaHCO_3$ solution, which, on addition of periodic acid and the sample for analysis (itself usually acid), maintained the pH of the reaction mixture at about 7 at least during the earlier stages of the aeration.

The aldehyde material obtained from *dl*-threonine was identified qualitatively as acetaldehyde by conversion into the 2:4-dinitrophenylhydrazone.

Yield of volatile aldehyde from other amino-acids

dl-Serine, under the same conditions, yielded no aldehyde in the absorption tower, nor did *dl*-alanine, *l*-cystine, *l*-methionine, *l*-hydroxyproline, *i*-β-hydroxyglutamic acid, *l*-tyrosine, *l*-tryptophan, *l*-arginine, *dl*-lysine or *l*-histidine treated separately under the same conditions.

Yield of acetaldehyde from dl-*threonine in the presence of other amino-acids*

From a mixture of 0·78 mg. of *dl*-threonine and 2·9 mg. of *dl*-serine (Hofmann) 70 % of the aldehyde calculated for the threonine present was obtained.

In order to control more rigidly the specificity of the determination before applying it to protein hydrolysates, a mixture of amino-acids was made up, as shown in Table 2, to simulate a wool hydrolysate. This mixture was refluxed

Table 2. *Mixture of amino-acids used in control experiments*

Amino-acid	N (mg.)	N as % of total N
Ammonia (as NH_4Cl)	26·4	5·3
dl-Alanine	32·4	6·4
l-Arginine (as hydrochloride)	96·9	19·4
l-Aspartic acid	11·7	2·3
l-Cystine	59·4	11·8
l-Glutamic acid	44·6	8·9
Glycine	83·0	16·5
l-Histidine (as hydrochloride)	9·0	1·8
dl-Leucine	51·8	10·3
dl-Lysine (as dihydrochloride)	22·3	4·4
l-Methionine	6·6	1·3
dl-Phenylalanine	12·8	2·6
l-Tryptophan	5·3	1·1
l-Tyrosine	16·2	3·2
dl-Valine	23·4	4·7
Total	501·8	100

for 24 hr. in $6N$ HCl, and then evaporated to dryness *in vacuo*; the residue was dissolved in water, and again evaporated to dryness. An aliquot of this *material A*, containing 40 mg. N, was treated with 200 mg. of periodic acid in excess of $NaHCO_3$ solution, and yielded only 0·44 μmol. of volatile aldehyde, equivalent (1 mol. aldehyde = 1 atom N) to 0·014 % of the N in the reaction mixture.

When 10·1 mg. of *dl*-serine were added to the same mixture, no additional aldehyde was recovered. The addition of 9·5 mg. of *dl*-threonine gave volatile aldehyde equivalent to 70 % of the threonine added.

Volatile aldehyde from protein hydrolysates

In view of the satisfactory character of the above control experiments, it was decided to apply the determination to protein hydrolysates. The HCl hydrolysate of about 30 mg. of protein (after removal of excess HCl by repeated evaporation with water *in vacuo*) was treated with 50 mg. of periodic acid in 25 ml. $M/5$ $NaHCO_3$ solution in the usual manner. The volatile aldehyde obtained from a number of proteins is shown in Table 5 as N in percentage of the total N of the protein. In the cases of wool, casein, gelatin and wheat gluten, the recovery of additional aldehyde from threonine added to the reaction mixture with the protein hydrolysate was 65–70 %, substantially the same as in the absence of the hydrolysate. The above conditions were therefore adopted as standard for the determination.

Effect of concentration of reagents on yield of aldehyde

Increasing the concentration of periodic acid tenfold had no effect on the yield of volatile aldehyde, while similar increases of concentration of both protein hydrolysate and periodic acid (i.e. 300 mg. protein and 500 mg. periodic acid in 25 ml. $M/5$ $NaHCO_3$) in the case of some proteins, though not of others, lowered the percentage yield of aldehyde. The most extreme lowering observed (30 %) occurred in the case of wool.

DISCUSSION

Although successful in differentiating threonine from other hydroxyamino-acids such as serine, β-hydroxyglutamic acid, 'hydroxylysine' etc. the method outlined above cannot be expected to differentiate threonine from its immediate higher homologues. For this reason, in view particularly of the *ad hoc* character of the authentication of threonine as the result of a research on the nutritive properties of amino-acids [McCoy *et al.* 1935], as well as the reported, though unconfirmed, isolations of higher hydroxyamino-acids [cf. Vickery & Schmidt, 1931; Dunn, 1938], it seemed to us desirable to investigate the qualitative composition of the volatile aldehyde material which we obtained from the various protein hydrolysates. Employing the conventional 2:4-dinitrophenylhydrazone identification (aeration into a saturated solution of 2:4-dinitrophenylhydrazine in $2N$ HCl, followed by recrystallization of the resulting precipitate from alcohol), we obtained, in the cases of wool, casein and gelatin, only the dinitrophenylhydrazone of acetaldehyde, which was identified by melting point, mixed melting point and X-ray powder photographs. In the case of wheat gluten, however, a mixture of the usual yellow crystals of acetaldehyde dinitrophenylhydrazone with redder crystals resembling those of the propaldehyde derivative was obtained. The X-ray powder photographs given by this mixture differed from those given by the three other preparations, and were closely similar, as pointed out by Dr F. O. Bell in an Appendix to this paper, to the photograph given by a mixture of about 3 parts of acetaldehyde dinitrophenylhydrazone to 1 part of the corresponding propaldehyde derivative. This conclusion was confirmed by the melting-point data, and in the preliminary note of this work [Martin & Synge, 1940] it was therefore suggested that a higher homologue of threonine, β-hydroxy*nor*valine, might be present in wheat gluten.

It seemed desirable to examine the matter quantitatively, and for this purpose we developed the method outlined below for the separation of acetaldehyde and propaldehyde on the micro scale.

MICRO-SEPARATION OF ACETALDEHYDE AND PROPALDEHYDE

The problem of separating successive members of a homologous series on the micro scale by physical means has not to our knowledge previously been attempted. It seemed to us that a simple method was available if it was possible to find a solvent of intermediate boiling point from which the substances to be separated could be differentiated by chemical means and for which Raoult's vapour pressure law was approximately obeyed. If a mixture of the two solutes were then allowed to reflux in excess of the solvent in an efficient fractionating column, the more volatile substance should be concentrated at the top of the column, while the less volatile should remain in the distilling vessel and the lower part of the column.

We accordingly set up the apparatus illustrated in Fig. 1, which incorporates a Lesesne & Lochte [1938] spinning band column, for which high efficiencies have been demonstrated by Baker *et al.* [1940]. The apparatus was made of pyrex glass; the spinning band was of aluminium and was driven through a pulley mounted on a Quickfit & Quartz bearing and gland with standard brass-glass conical joint. The column was about 1½ m. long, and was lagged with wool. The material for analysis was aerated into the solvent in the distilling vessel, which was cooled with solid CO_2, thus trapping the aldehydes. The distilling vessel was then placed in a water bath and the solvent was allowed to reflux with the band spinning at about 1000 r.p.m. while a current of air was drawn through the apparatus at a controlled rate of 120 bubbles per min. The air from the condenser passed to the absorption tower containing $NaHSO_3$, the aldehyde trapped being determined as already described.

As the boiling points of acetaldehyde and propaldehyde are 21° and 49° respectively, ether (B.P. 37°) seemed the most suitable solvent to employ. However, commercial ether contains appreciable quantities of aldehyde, and ether freed from aldehyde by the procedure of Carey *et al.* [1933] (washing with 30% $NaHSO_3$ followed by 10% NaOH) was found to contain aldehyde again after standing in the laboratory for a few days.

In view of this we tried to use light petroleum which had been fractionated in an efficient distilling column (B.P. 34°). This did not give rise to aldehyde. With petroleum in the column, however, both acetaldehyde and propaldehyde were recovered quantitatively in the absorber, as both appear to form low-boiling azeotropes with petroleum.

As Hildebrand [1921] has shown thermodynamically, deviation from Raoult's law is to be expected whenever two liquids on mixing show volume or heat changes. Liquids are likely to obey Raoult's law when the ratios $\frac{\text{temperature coefficient of expansion}}{\text{compressibility}}$ are similar for both pure liquids and their mixtures. This ratio is a measure of the polarity of the liquids. Lecat [1930; 1932] has made an extensive empirical study of the phenomenon of azeotropy in binary mixtures, and has summarized the literature, but we have not been able to find a treatment of the phenomenon in terms of molecular physics. It is clear, however, that azeotropy is to be expected in mixtures of liquids of unequal polarity and is related to the formation of separate liquid phases. The use of azeotropic distillation in the manufacture of absolute alcohol is well known. Recently Matuszak & Frey [1937] have used distillation with SO_2 for the separation of butane isomers from butene isomers; Schicktanz *et al.* [1939] have used CCl_4 for the separation of the lower aliphatic alcohols in fusel oil, and [1940] benzene and toluene for the separation of the lower fatty acids. It is evident that the extreme form of azeotropic distillation is the distillation of two liquid phases, as in the steam distillation of oils, and that azeotropic distillation depends not only on the volatility but also on the polarity of the substances to be separated, and thus offers some of the advantages of liquid extraction.

Owing to the azeotropy of the system propaldehyde-petroleum we were compelled to use ether as solvent; when this had been extracted with $NaHSO_3$ followed by NaOH it gave satisfactory blanks for aldehyde on distillation, although it proved impossible to estimate the propaldehyde remaining in the ether in the apparatus by direct extraction of this with $NaHSO_3$ solution, since, with ether, an aldehyde titration was always obtained on shaking with the solution in a separating funnel, and this increased as the extraction was prolonged. We were therefore forced to estimate propaldehyde etc. remaining in the apparatus by the difference between the aldehyde estimated by direct aeration and by aeration through the column. We obtained positive evidence for its presence by adding petroleum to the ether in the apparatus after the acetaldehyde had been removed. In the time employed about two-thirds of the propaldehyde present was in this way collected at the top of the column.

The results of the control experiments are shown in Table 3.

Table 3. *Aldehyde micro-separation. Control experiments*

	Aldehyde taken in μmol.		Aldehyde found in μmol.	
Exp.	Acetaldehyde	Propaldehyde	A	B
I	0·0	0·0	0·0	0·5
II	12·9	28·0	14·8	—
III	26·4	0·0	26·6	0·5
IV	0·0	29·5	0·8	17·0
V	26·0	1·3	29·6	1·6

In these experiments the aldehydes were first collected in 100 ml. of aldehyde-free ether cooled with solid CO_2. The ether was then allowed to reflux in the column while a stream of air was drawn through the apparatus for 1 hr. at a rate of 120 bubbles per min. in the wash-bottle (Fig. 1), which contained water. At the end of this period the aldehyde collected in the absorber was titrated, giving the values shown in column A of Table 3. 100 ml. of aldehyde-free low-boiling petroleum were then added to the distilling vessel and aeration was continued for 2 hr. The aldehyde in the absorber was then again titrated, giving the values shown in column B. It will be seen from Exp. IV that about two-thirds of the propaldehyde present is carried up in the second stage of the distillation, and from comparing Exps. III and V that as little as 5 % of propaldehyde in 1 mg. of acetaldehyde may readily be detected.

A control experiment with *iso*valeraldehyde showed that it did not reach the top of the column even after adding the petroleum; however, the autoxidation of aliphatic aldehydes higher than propaldehyde renders this analytical procedure unsuitable if a stream of air is employed.

The experiments with protein hydrolysates are shown in Table 4. Here the HCl hydrolysate of about 0·3 g. of protein was used, freed from excess of HCl by evaporation *in vacuo* and treated with 300 mg. of periodic acid in 10 ml. sat. aqueous $NaHCO_3$ solution. This mixture was aerated, and the volatile aldehyde was collected in cooled ether as already described. The experiments were then carried out exactly as the controls.

Table 4

Protein	Plant	Natural order	Vol. aldehyde as N in % of total N (cf. Table 5)	μmol. aldehyde found	
				A	B
Gluten	Wheat (*Triticum vulgare*)	Gramineae	1·2	26·2	0·7
Zein	Maize (*Zea Mays*)	Gramineae	1·2	26·9	0·9
Leaf protein	Cocksfoot (*Dactylis glomerata*)	Gramineae	2·2	47·6	0·8
Seed globulin	Peanut (*Arachis hypogaea*)	Leguminosae	1·1	36·0	1·0
Seed globulin (Edestin)	Hemp (*Cannabis sativa*)	Urticaceae	1·7	30·2	0·6
Seed globulin	Castor bean (*Ricinus communis*)	Euphorbiaceae	1·5	35·9	0·7

The results make it unlikely that in any case more than 3 % of the aldehyde material was higher aldehyde than acetaldehyde. In each case rather more aldehyde was obtained as in column A than by direct aeration from the reaction mixture into the absorption vessel. There is thus no evidence for the occurrence in these proteins of higher homologues of threonine.

Discussion

Our earlier report of propaldehyde was based on melting-point and X-ray data on the dinitrophenylhydrazone material derived from wheat gluten. It subsequently came to our notice that acetaldehyde 2:4-dinitrophenylhydrazone is dimorphic [cf. Bryant, 1933]: this has been questioned by Campbell [1936]. The metastable form, obtained from the melt or by rapid evaporation from solution, bears a close resemblance to propaldehyde dinitrophenylhydrazone being decidedly more deeply coloured (red) than the stable form (yellow). In the light of this, we found that if authentic acetaldehyde and dinitrophenylhydrazine were allowed to react in HCl solution through which air from the reaction mixture with periodic acid of a wheat gluten hydrolysate had been drawn for sufficiently long to remove completely all acetaldehyde, propaldehyde etc. the resulting dinitrophenylhydrazone had a tendency to crystallize from alcohol in the metastable form even when crystallization was slow. It seems therefore that a trace of some slightly volatile contaminant promotes, in the case of the material from wheat gluten (not with casein, wool or gelatin), crystallization of the acetaldehyde derivative in the metastable form.

The dinitrophenylhydrazone material derived from wheat gluten, on chromatographic analysis in petroleum solution on MgO, gave two fractions very readily—a small proportion of red or brown material being very strongly adsorbed. The material obtained after the removal of this component showed, however, the same tendency to crystallize in the metastable form. The development of the chromatogram was not carried sufficiently far to permit of the separation of the acetaldehyde and propaldehyde derivatives, though control experiments suggested that this separation can be effected chromatographically; this requires long development, and the bands become too diffuse for the satisfactory visual detection of a small proportion of one component.

The various dinitrophenylhydrazone materials have been investigated by Dr F. O. Bell by the X-ray powder method; she presents her findings in an Appendix to this paper.

We conclude: (1) that the amount of propaldehyde obtained in this way from wheat gluten is at most small; (2) that acetaldehyde 2:4-dinitrophenylhydrazone, a compound much used by biochemists for the formal identification of acetaldehyde, has disadvantages for this purpose because of its dimorphism, which is now thoroughly substantiated by the X-ray work.

Criticism of the new procedure

The method for the determination of threonine, outlined above, is compared here with the method of Block & Bolling [1939].

(1) It requires the use of rather more material, though it remains on the micro scale.

(2) It is more rapid and less laborious. In particular it does not necessitate the transfer of material to an anhydrous solvent; the reaction is carried out in aqueous solution.

(3) The method has been shown to have a high specificity, threonine being the only known protein constituent to give volatile aldehyde under the conditions used for the determination. Block & Bolling report with their method interference by alanine and serine.

(4) It seems probable that higher homologues of threonine, if present, would be estimated by the procedure described here, though not by the procedure of Block & Bolling. To detect the presence of these we have developed the micro-separation technique described above, and the results suggest that higher homologues of threonine are absent from a number of proteins. We should like to emphasize the applicability of the type of separation described here for aldehydes to micro-separations in other homologous series. In its simplest form, a carrier of intermediate boiling point is required, from which the substances under analysis may be separated and determined by chemical means. It is necessary that at least one, and preferably both, of the substances to be separated should not show positive azeotropy with the solvent. (For separating substances of *different* chemical classes on the micro scale by distillation, 'carrier' solvents showing azeotropy with one class but not with the other may greatly facilitate separation, as has been shown by Matuszak & Frey [1937], and in this case it is not always necessary for the solvent to be of intermediate boiling point.)

(5) The yield of acetaldehyde obtained from threonine by our procedure seems to be about 70% of the theoretical and to be largely independent of the presence of other amino-acids. It is impossible to deduce from Block & Bolling's paper what yield of acetaldehyde was obtained by them, since they used lithium lactate for checking the method; they found lithium lactate to give, under the same conditions as threonine, the same yield of acetaldehyde. Macara & Plimmer [1940] have recently reported experiments using the method of Block & Bolling. They found certain modifications of the procedure to be necessary, and failed to obtain very consistent results.

2. DETECTION AND ISOLATION OF FORMALDEHYDE LIBERATED BY PERIODIC ACID

We have not so far found a satisfactory method for determining the formaldehyde resulting from the action of periodic acid on serine etc.

Nicolet & Shinn [1939] obtained a good yield of formaldehyde through the dimedone derivative [Vorländer, 1929], after acting on serine with periodic acid, and, as shown below (Exp. I), we can confirm this result. The yield, however, is substantially reduced in the presence of a second amino-acid, such as tyrosine, which does not react with periodate, as shown in Exp. II, while in a complex mixture of amino-acids of known composition (Exp. III) no precipitation of formaldehyde-dimedone takes place. This can be ascribed to competition between the dimedone and the amino-acids for combination with the formaldehyde; in view of Exp. III it is not surprising that a wool hydrolysate, after treatment with periodate and removal of acetaldehyde by aeration, gave no precipitate on treatment with dimedone.

Nevertheless, though the dimedone method seems valueless for determining serine etc. in complete protein hydrolysates, it has proved of some value, as shown in Sections 4 and 5 of this paper, in setting lower limits to the purity of preparations derived from protein containing a high proportion of those amino-acids that yield formaldehyde on treatment with periodic acid.

EXPERIMENTAL

Exp. I. 10·8 mg. of *dl*-serine were treated with 50 mg. of periodic acid in 25 ml. of *M*/5 $NaHCO_3$ at room temperature. After a few minutes, 60 mg. of dimedone (B.D.H.) were added. When the dimedone had dissolved, the mixture was acidified with acetic acid, and after an hour the resulting precipitate was filtered off, washed and dried. It weighed 26·9 mg., corresponding to a yield of 90 % of 1 mol. of formaldehyde per mol. of serine.

Exp. II. 10·1 mg. of *dl*-serine were treated exactly as in Exp. I except that 24·5 mg. of *l*-tyrosine were also present in the reaction mixture. The resulting precipitate weighed 9·4 mg., corresponding to a yield of 33 % of 1 mol. of formaldehyde per mol. of serine.

Exp. III. A portion of *material A* (cf. Section 1) containing altogether 40 mg. N was added to 10·1 mg. of *dl*-serine and was treated with 200 mg. of periodic acid in the presence of excess of $NaHCO_3$ solution. (Volume of reaction mixture, 27 ml.) On adding dimedone and acetic acid as in Exps. I and II, no precipitate resulted.

3. AMMONIA SPLIT FROM HYDROXYAMINO-ACIDS BY PERIODIC ACID

Van Slyke *et al.* [1940] reported that 1 mol. of NH_3 is instantaneously set free from serine, threonine, β-hydroxyglutamic acid and 'hydroxylysine' by periodic acid in strong aqueous K_2CO_3 solution. They determined the NH_3 liberated by the aeration method of Van Slyke & Cullen [cf. Peters & Van Slyke, 1932]. Macara & Plimmer [1940] have recently employed this method for the determination of amino-acids of this type in hydrolysates of fractions derived from casein. They too report quantitative recovery of NH_3, although details of the experimental conditions are not given. Since the present work was carried out, we have received fromDr Van Slyke, in a private communication, details of his procedure; this involves the use in the reaction mixture of NaOH as well as K_2CO_3; a reagent is also added to prevent, by competition, condensation of NH_3 with the aldehydes also formed in the reaction. Nevertheless, while the experiments described here are quantitatively imperfect, they serve to demonstrate the specificity of the method, and the results obtained with protein hydrolysates are at least of comparative significance.

EXPERIMENTAL

In these experiments we used simple aeration tubes (as shown in Fig. 1) without the perforated bubblers of Van Slyke & Cullen. Absorption of ammonia in the standard acid was found to be 92 %; all the results obtained below have been corrected for this loss. This cruder apparatus seems to have an accuracy of about ± 5 %, but in view of the low yields of NH_3 we were content with this.

Exp. I. 5·92 mg. of *dl*-serine in 10 ml. of 50 % w/v aqueous K_2CO_3 solution with 50 mg. of periodic acid gave on aeration at room temperature 88 % of 1 mol. of NH_3. Under the same conditions, equivalent quantities of the following amino-acids gave the yields of NH_3 stated: threonine, 83 %; *dl-allo*threonine, 86 %; *i*-β-hydroxyglutamic acid, 71 %.

Exp. II. An amount of *material A* (cf. Section 1) containing 7·6 mg. N was added (as 0·5 ml. of aqueous solution) to 8 ml. of 62 % w/v aqueous K_2CO_3. The ammonia set free was removed by aeration, and was found to be 102 % of the amount calculated on the basis of the NH_4Cl used in making up this mixture.

100 mg. of periodic acid were then added, and the aeration was continued. No further ammonia was detected in the acid of the absorption vessel.

Exp. III. Exp. II was repeated, adding known quantities of hydroxyamino-acids to the mixture before carrying out the determination. On adding 5·92 mg. of *dl*-serine, 72 % of 1 mol. of NH_3 was recovered on treatment with periodic acid; on adding 7·6 mg. of threonine, 77 % of 1 mol. of N_3 was recovered.

Table 5. *Hydroxyamino-acid determinations on various protein hydrolysates*

Protein	N % of protein	As % of total N of protein 'Amide-N'	NH₃-N lib. by periodate	'Threonine-N' (uncorrected)
Wool: Merino 64's I	16·7	8·2	9·2	3·3
Merino 64's II	—	—	—	3·5
Cotswold*	16·55	8·7	9·2	3·0
Commercial pure wool flannel	16·6	8·3	9·9	2·8
Silk fibroin	18·3	2·2	9·4	0·4
Myosin (68)†	16·8	7·5	4·3	1·9
Myosin (KB)†	16·8	7·9	4·5	2·0
Myosin (X)†	16·8	7·4	4·1	1·9
Myogen (C_2)†	16·8	7·0	5·4	2·4
Myogen (D)†	16·8	5·7	5·3	1·9
Gelatin (Coignet Gold Label)	—	—	3·4	1·0
Casein B.D.H.	13·4	10·4	6·3	2·1
Egg albumin	15·8	7·9	7·2	1·6
Wheat gluten B.D.H.	13·5	23·3	5·3	1·2
Zein	16·3	19·7	5·9	1·2
Leaf protein: *Dactylis glomerata*	13·1‡	7·5	5·4	2·1
Seed globulin: *Arachis hypogaea*	—	12·3	5·0	1·1
Cannabis sativa	18·55	11·0	5·4	1·7
Ricinus communis	—	11·1	5·3	1·5

* Before analysis cystine had been removed from the hydrolysate by Cu_2O.

† These preparations of rabbit-muscle proteins were provided by Dr J. G. Sharp of the Low Temperature Research Station, Cambridge. Myosin KB is a sample of myosin reprecipitated six times.

‡ On ash-free basis: the preparation contained 2·4 % of ash.

On the basis of these experiments, we have adopted the following routine method for the estimation of the hydroxyamino-acid content of complete protein hydrolysates:

An amount of an HCl hydrolysate (from which excess of HCl has been removed by repeated evaporation with water *in vacuo*), corresponding to 60–70 mg. of protein and not exceeding 2 ml. in volume, is placed in the aeration tube with 8 ml. 62 % w/v aqueous K_2CO_3 solution, and the 'amide-N' is determined by aeration into standard acid. 100 mg. of periodic acid are then added, and the aeration is repeated. The ammonia thus set free is taken as a measure of the hydroxyamino-acids of this type present. In most cases rapid aeration was not interfered with by frothing of the reaction mixture.

In Table 5 are set out parallel determinations of 'ammonia-N', 'N liberated by periodate' and 'threonine-N' as percentages of the total N of the proteins, as determined by the Kjeldahl procedure.

Discussion

All the indications are that the 'threonine' values set out in Table 5 are 30–35 % low (cf. Section 1) and the 'N liberated by periodate' 20–30 % low. The results are, at least, of comparative significance, and set lower limits to the amount of the amino-acids in question that are present.

In the cases of wool, casein, gelatin, gluten, cocksfoot leaf protein and the seed globulins of *Arachis*, *Cannabis* and *Ricinus*, there is good evidence that the volatile aldehyde set free is very largely acetaldehyde, and therefore probably derived from threonine (cf. Section 1).

The high content of hydroxyamino-acids in wool and silk fibroin, compared with the other proteins studied, is to be noted. In the case of wool, about one-third of this quantity appears to be threonine: silk fibroin, however, appears to have the lowest threonine content of all the proteins investigated. In each case, the difference between these values is probably mostly due to serine—in which silk fibroin is known to be comparatively rich [Fischer & Skita, 1902; Abderhalden 1922]. In the present work (Section 4) it is shown that wool also contains considerable quantities of serine.

Decomposition of a proportion of this serine may be expected to account for part of the ammonia found in the acid hydrolysates of both these proteins [cf. Damodaran & Ramachandran, 1941].

From Table 5 zein would be expected to be fairly rich in serine. This is in agreement with the isolation from zein by Osborne & Jones [1910] of 1 % of serine.

4. THE HYDROXYAMINO-ACID FRACTION OF WOOL

In this section we report the results of an investigation, using the analytical procedures developed above, of the fraction obtained from a wool hydrolysate by the acetylation-benzoylation procedure of Synge [1939]. In that paper, a fraction from wool was reported containing 5·3 % of the N of the wool (96 % of it amino-N). In a subsequent paper [Martin & Synge, 1941] we described a similar preparation (made after removal of the higher monoamino-acids from the hydrolysate by acetylation and chloroform extraction) containing 5·9 % of the N of the wool (95 % of it amino-N). This latter material, obtained as *N*-acetyl derivatives in the form of a nearly colourless glass, was employed in the present investigation.

EXPERIMENTAL

Some of the *N*-acetylated material was hydrolysed by refluxing for 3 hr. in 15 ml. of 2*N* H_2SO_4. This contained 122·5 mg. N, and all values given below refer to this original quantity, being corrected for aliquots removed for analysis.

$SO_4^=$ was then exactly removed with baryta, and the filtrate and washings from $BaSO_4$ were made up to standard volume. A threonine determination (cf. Section 1) on an aliquot of this material gave the threonine-N (uncorrected) as 9·2 % of the N of the fraction, i.e. 0·54 % of the total N of the wool.

The rest of the solution was concentrated by evaporation *in vacuo* and deposited crops of insoluble crystalline material, which were removed by filtration (tyrosine). This material amounted in all to 323 mg., equivalent to 25·0 mg. N, i.e. 20 % of the N of the fraction and 1·2 % of the N of the original wool.

The aqueous filtrate from tyrosine on addition of alcohol gave a syrup which slowly crystallized. These crystals were recrystallized from water-alcohol several times; after two crystallizations the threonine content (uncorrected) of the crystalline material had fallen to about 1 %, at which value it remained on further recrystallization.

The final product (*material S*) weighed 330 mg.

Analytical data on material S

Elementary analysis. Found: C, 31·8; H, 6·35; N, 12·4; ash, 6·6 %. The product therefore contained 40·8 mg. N, i.e. 33 % of the N of the hydroxyamino-acid fraction and 1·95 % of the total N of the wool. 'Corrected' for ash content, the analytical figures become: C, 34·1; H, 6·81; N, 13·3 % (calc. for serine, $C_3H_7O_3N$: C, 34·3; H, 6·66; N, 13·3 %).

Optical rotation. Material S had $[\alpha]_D^{17\cdot5^\circ} -8\cdot06^\circ$ (water, $c=5$). Fischer & Jacobs [1906] record *l*-serine as having $[\alpha]_D^{20^\circ} -6\cdot83^\circ$ (water, $c=10$).

Formaldehyde on treatment with periodate. On treating 13·5 mg. of S with periodate and dimedone, as described in Section 2, 25·4 mg. of formaldehyde dimedone were obtained. On recrystallization this material had M.P. 188–189°, not depressed on admixture of authentic formaldehyde dimedone, for which Vorländer [1929] records M.P. 189°. (Found: C, 70·5; H, 8·33 %. Calc. for $C_{17}H_{24}O_4$: C, 69·9; H, 8·23.)

The yield of formaldehyde was thus equivalent to 73 % of the N of the material.

Ammonia on treatment with periodate. 7·2 mg. of *material S* gave, on treatment with K_2CO_3-periodate as in Section 3, 88 % of its N as NH_3—the same yield as that obtained from pure serine under the same conditions.

Material S gave a negative orcin-H_2SO_4 test for carbohydrate.

Discussion

From the above analytical data we conclude that *material S* consists largely of serine, contaminated by inorganic material. The fact that its optical rotation is significantly higher than that reported for *l*-serine by Fischer & Jacobs may be ascribed either to a contaminant of high laevorotation or to a raising of the rotation produced by the inorganic material present. It seems probable, nevertheless, that the acetylation-benzoylation procedure affords a method for isolating serine from proteins in the optically active state. This has not often been reported since Fischer [1907] obtained his active product from silk [cf. Posternak & Posternak, 1927; Lipmann & Levene, 1932].

The results of the present work on the hydroxyamino-acid fraction of wool are summarized in Table 6.

Table 6. *Composition of the hydroxyamino-acid fraction prepared from wool*

		N as % of N of fraction	N as % of N of wool
Identified as:	serine	33	1·95
	threonine (uncorrected)	9·2	0·54
	tyrosine	20	1·2
Unidentified		37·8	2·2
	Total	100	5·9

It seems likely that a large proportion of the unidentified N is serine, as unavoidable losses were encountered in working up *material S*.

By making use of the figures given in Table 5 for the original wool hydrolysate (Merino 64's I) it is possible to form an estimate of the yields obtained by the acetylation-benzoylation procedure.

The threonine content of the original hydrolysate (corrected—assuming the figure in Table 5 to be low by 30 %) is 4·7 % of total N. The threonine present in the hydroxyamino-acid fraction (from Table 6, corrected in the same way) is only 0·77 % of the total N of the wool. This represents a recovery, in the hydroxyamino-acid fraction, of 16 % of the threonine present in the hydrolysate.

The recovery of serine may be estimated as follows: assuming that the 'N liberated by periodate' as given in Table 3 is 25 % low (cf. Section 3), we have 'N liberated by periodate' as 12·2 % of the total N of wool. Subtracting the 'corrected' value for threonine-N of 4·7 %, this leaves a maximum value for serine-N in the original hydrolysate of 7·5 % of the total N. In the hydroxyamino-acid fraction, the upper limit for serine is (total N − threonine N (corrected) − tyrosine N) = 4·0 % of total N of wool (cf. Table 6). On this basis, a recovery of about 50 % of the serine of the original hydrolysate is obtained in the hydroxyamino-acid fraction.

This difference between the behaviours of serine and threonine may perhaps be ascribed to a more ready benzoylation of the primary alcoholic group of serine than of the secondary alcoholic group of threonine, in the course of preparing the hydroxyamino-acid fraction.

Nevertheless, in hydroxyproline, as shown by Synge [1939], the secondary alcoholic group is readily benzoylated, as is the phenolic —OH group of tyrosine.

5. 'HYDROXYLYSINE'

Van Slyke & Hiller [1921] reported the occurrence of a base of unknown constitution in the lysine fraction of a gelatin hydrolysate. Schryver *et al.* [1925] reported the isolation of a new base from isinglass, of which they prepared a tribenzoyl derivative. They suggested that it was a hydroxy derivative of lysine, and tentatively assigned the —OH group to the β-position, by analogy with serine and β-hydroxyglutamic acid. Van Slyke *et al.* [1938] reported the isolation of a base from the lysine fraction of a gelatin hydrolysate by fractional crystallization of the picrates; it had the composition of hydroxylysine and its picrate had M.P. 225°. More recently Van Slyke *et al.* [1940] have analysed the electrometric titration curve of the new base, and have shown that the compound is split by periodic acid to give one molecule of formaldehyde and one molecule of ammonia. They suggest that the new base is either δ-hydroxy-αε-diaminohexoic acid or ε-hydroxy-αδ-diaminohexoic acid.

Shortly after the 1938 note of Van Slyke *et al.* one of us (R. L. M. S.) applied the acetylation-benzoylation procedure of Synge [1939] to the lysine fractions of gelatin and isinglass hydrolysates and in each case isolated a picrate corresponding to the description given by Van Slyke *et al.* [1938].

EXPERIMENTAL

Approximately 150 g. of gelatin (Coignet Gold Label) containing 21·2 g. N were hydrolysed by refluxing with 1·5 l. of 6*N* H_2SO_4 for 6 hr. The hydrolysate was cooled and treated with 300 g. of phosphotungstic acid (B.D.H.) at a final volume of 5 l. After keeping overnight in the ice-chest, the precipitate was filtered off and decomposed with baryta as usual. The filtrate and washings from the decomposition were concentrated *in vacuo* to remove NH_3, acidified to thymol blue with H_2SO_4 and treated with Ag_2SO_4 at 100° until excess had been added (brown precipitate on testing a portion with baryta). The solution was cooled, saturated with $Ba(OH)_2$ and filtered, and the filtrate, after acidification to thymol blue with H_2SO_4 (to remove Ba), was treated with H_2S to remove Ag. The filtrate from Ag_2S and $BaSO_4$ was freed from H_2S by concentration *in vacuo* and was then treated with 20 ml. of H_2SO_4 and 100 g. of phosphotungstic acid at a volume of 1·5 l. After keeping overnight in the ice-chest, the precipitate was filtered off and decomposed with baryta as usual. The filtrate was acidified with H_2SO_4 to precipitate Ba, and the filtrate from $BaSO_4$, the 'lysine fraction', was found to contain 4·3 % of the original N of the gelatin in the form of amino-N (½ hr. Van Slyke determination). This solution was then treated with two-thirds of the theoretical quantity of picric acid, and the resulting lysine picrate, after recrystallization from water, corresponded to 1·93 % of the total N of the original gelatin. The combined mother liquors and washings from this were freed from picric acid by acidification with H_2SO_4 and repeated extraction with chloroform. The aqueous residue was then brought to *p*H 8·5 with NaOH, concentrated to small volume *in vacuo* and subjected to the acetylation-benzoylation procedure [Synge, 1939], employing for the acetylation 200 ml. 2*N* NaOH and 20 ml. of acetic anhydride and for the benzoylation 185 ml. 2*N* NaOH and 23 ml. of benzoyl chloride. The resulting *N*-acetylated material was hydrolysed in 108 ml. 2*N* H_2SO_4 by refluxing for 3 hr., after which the solution was found to contain 57·5 mg. amino-N (½ hr. Van Slyke determination) equivalent to 0·27 % of the total N of the gelatin. 20 g. of phosphotungstic acid were added in 60 ml. of water. Precipitation of the resulting phosphotungstate, a fluffy, crystalline material (which did not readily settle, but filled the body of the solution), was slow, but had ended after keeping overnight in the ice-chest. The precipitate was filtered off and decomposed in the usual manner with baryta; the amino-N obtained was found (½ hr. Van Slyke determination) to correspond to 0·13 % of the total N of the gelatin.

This material yielded with picric acid 121 mg. of a crystalline picrate, which on recrystallization from water had explosion point 226–227° and amino-N (½ hr. Van Slyke determination) 7·38 %. ($C_6H_{14}O_3N_2 . C_6H_3O_7N_3$ requires amino-N 7·17 %.)

A picrate having the same properties was obtained in the same way from isinglass (Boots 'Besyet'); the product resulting from decomposition of the final phosphotungstic acid precipitate contained amino-N equivalent to 0·15 % of the original N of the isinglass.

Several preparations obtained in this way were combined, and after the appearance of the 1940 note of Van Slyke *et al.* it seemed to us desirable to test the material for formaldehyde and ammonia on treatment with periodate.

A control experiment with synthetic *dl*-lysine [cf. Eck & Marvel, 1939] showed that on treatment with periodate lysine produces neither of these substances.

Formaldehyde liberated by periodate from the base. 57·3 mg. of the combined picrate preparations were freed from picric acid by extraction with chloroform from 10 ml. of 0·15 *N* HCl. The aqueous residue was freed from chloroform by aeration, and treated with 1 g. of $NaHCO_3$ and 120 mg. of periodic acid at a volume of 30 ml.; 120 mg. of dimedone were then added, and when it was dissolved the mixture was acidified with acetic acid. After keeping 1·5 hr. the resulting precipitate was filtered off, washed and dried. It weighed 28 mg., corresponding to a yield of 65 % of 1 mol. of formaldehyde per mol. of 'hydroxylysine'. On recrystallization from alcohol the material had M.P. 187–188°, which was not depressed on admixture of authentic formaldehyde-dimedone. (Found: C, 69·6; H, 8·14 %. Calc. for $C_{17}H_{24}O_4$: C, 69·9; H, 8·23 %.)

Ammonia liberated by periodate from the base. After removal of picric acid as above, followed by removal of chloroform by aeration, an amount of the base corresponding to 16·8 mg. of picrate gave on treatment with K_2CO_3 and periodic acid as in Section 3 0·19 mg. of NH_3-N. This is equivalent to the liberation of 31 % of a mol. of NH_3 per mol. of 'hydroxylysine'.

Discussion

It will be seen that the above experiments confirm the discovery of Van Slyke and co-workers although the yields of formaldehyde and ammonia fall short both of the 'theoretical' and of those obtained with serine and threonine under the same conditions. The method employed for the isolation of the base is in itself an indication that the base possesses a free hydroxyl group.

It seemed of interest to obtain an estimate of the amount of the base possibly present in gelatin. A sample of the 'lysine fraction' obtained as described above from Coignet Gold Label gelatin was treated with K_2CO_3 and periodic acid as in Section 3. 0·20 % of the total N of the gelatin was obtained as ammonia, suggesting that at least 0·4 % of the total N of the gelatin is in the form of 'hydroxylysine'. In view of the low yield of NH_3 obtained in this way from our purest preparation of the base, the amount actually present may be considerably greater.

It appears therefore that the yield of 'hydroxylysine' isolated by the acetylation-benzoylation procedure from the 'lysine fraction' is not high (< 33 %), in spite of the enormous excesses of reagents which it was possible to employ. This, by analogy with the behaviour of serine and threonine (cf. Section 4) is perhaps an indication in favour of the alcoholic group being secondary, i.e. favours the view that 'hydroxylysine' is δ-hydroxy-αε-diaminohexoic acid rather than ε-hydroxy-αδ-diaminohexoic acid.

A parallel experiment on the 'lysine fraction' of wool (Merino 64's I) prepared in exactly the same manner gave < 0·01 % of the total N of the wool in the form of NH_3 liberated by K_2CO_3 periodate.

In a private communication Dr Van Slyke informs us that the hydroxylysine-N in gelatin and wool amounts respectively to 0·9 and 0·11 % of the total N (minimal figures based on N liberated by periodate from recrystallized phosphotungstic acid precipitates).

The formula suggested for hydroxylysine by Van Slyke and co-workers may be said to have a biological justification. The idea that proline may have the same biological origin as the basic amino-acids was held by its discoverer [Fischer, 1903]. Wada's [1933] demonstration of the conversion of citrulline into proline on boiling with acid lends support to this hypothesis (cf. also Sørensen's [1905] conversion of δ-hydroxy-α-aminovaleric acid into proline). It is not likely that this reaction occurs to any great extent during the acid hydrolysis of proteins, although since the work of Wada [1933] and Fearon [1939] citrulline is to be regarded as a probable constituent of proteins, and its breakdown during acid hydrolysis would account for the small amounts of CO_2 that are set free [cf. Dunn, 1925] as well as for some NH_3. The balance of evidence suggests that proline residues exist pre-formed in proteins. If, however, their biological precursor is to be regarded as an ornithine derivative, then the precursor of hydroxyproline would be a derivative of γ-hydroxyornithine, which is homologous with δ-hydroxylysine. On this view it is to be expected that the asymmetry of the γ-C atom of hydroxyproline is the same as that of the δ-C atom of hydroxylysine. Neither configuration has yet been ascertained. In this connexion it is of interest that Felix & Mager [1937] report the occurrence of small amounts of hydroxyproline in clupein, together with still smaller amounts of a base having the elementary composition of hydroxyarginine. In the present work it is shown that hydroxyproline and hydroxylysine, both present in a gelatin hydrolysate, can only be present in small quantity in wool.

SUMMARY

1. Threonine on treatment with periodate at pH 7 yields 70 % of 1 mol. of acetaldehyde. Acetaldehyde and higher homologues, unlike formaldehyde, may be removed from aqueous solution by simple aeration; this provides the basis of a micro-method for the determination of threonine and higher homologues in complete protein hydrolysates and other material of biological interest. The determination is not interfered with by serine, 'hydroxylysine' or other known naturally occurring amino-acids.

2. The aldehyde material obtained in this way from a number of proteins has been shown to consist almost entirely of acetaldehyde. We have therefore no evidence for the occurrence of higher homologues of threonine in these proteins.

3. To demonstrate this, we have developed a micro-method for the separation of acetaldehyde from its higher homologues, which depends on 'carrier' distillation using ether as a solvent. By this means as little as 5 % of propaldehyde can be detected in 1 mg. of acetaldehyde. The principles of this type of separation, and its applicability to micro-separations in other homologous series, are discussed.

4. The explanation of our earlier report [Martin & Synge, 1940] of propaldehyde in the aldehyde material from wheat gluten appears to lie in the dimorphism of acetaldehyde dinitrophenylhydrazone. In an appendix, Dr Bell discusses the relevant X-ray data.

5. The conditions for precipitation by dimedone of the formaldehyde set free from serine etc. by periodic acid have been studied. The presence of other amino-acids seriously lowers the yield obtained.

6. The production of NH_3 by periodate from such hydroxyamino-acids as serine, threonine, β-hydroxyglutamic acid and hydroxylysine in the presence of K_2CO_3 [cf. Van Slyke *et al.* 1940] has been shown by control experiments to be

applicable to the estimation of the aggregate of these substances in complete protein hydrolysates.

7. The results of threonine and aggregate hydroxyamino-acid estimations on a number of proteins are tabulated and discussed.

8. Using the methods here developed, the hydroxyamino-acid fraction obtained from a wool hydrolysate by the acetylation-benzoylation procedure of Synge [1939] has been investigated. This procedure appears to give low recoveries of threonine. Nearly 2 % of the N of wool was isolated in the form of fairly pure serine showing optical activity.

9. A preparation agreeing in properties with the 'hydroxylysine' of Van Slyke *et al.* has been obtained from the lysine fractions of gelatin and isinglass hydrolysates by the acetylation-benzoylation procedure. This material, like Van Slyke's, yields ammonia and formaldehyde on treatment with periodic acid.

We wish to thank Dr Bell and her colleagues for the X-ray investigations, and for the interest which they have taken in this work. We are also grateful to Dr K. Bailey for a de-cystinized hydrolysate of Cotswold wool, to Dr H. E. Carter for a sample of *O*-methyl-*dl*-*allo*threonine, to Prof. A. C. Chibnall for synthetic threonine and specimens of various proteins, to Prof. C. R. Harington for synthetic β-hydroxyglutamic acid, to Dr J. G. Sharp for specimens of natural threonine and of various muscle proteins, to Miss Thornton and Mr Warburton for preparing the figure, and to Miss M. I. Whitehead for help in preparing the paper for press.

Finally we wish to express our gratitude to Prof. A. C. Chibnall, Sir C. J. Martin, Mr N. W. Pirie and Mr B. H. Wilsdon for their interest and encouragement.

REFERENCES

Abderhalden (1922). *Hoppe-Seyl. Z.* **120**, 207.
Baker, Barkenbus & Roswell (1940). *Industr. Engng Chem.* Anal. ed. **12**, 468.
Blair & Ledbury (1925). *J. chem. Soc.* p. 26.
Block & Bolling (1939). *J. biol. Chem.* **130**, 365.
Bryant (1933). *J. Amer. chem. Soc.* **55**, 3201.
Campbell (1936). *Analyst*, **61**, 391.
Carey, Green & Schoetzow (1933). *J. Amer. pharm. Ass.* **22**, 1237.
Cramer (1865). *J. prakt. Chem.* **96**, 76.
Criegee, Kraft & Rank (1933). *Liebigs Ann.* **507**, 159.
Damodaran & Ramachandran (1941). *Biochem. J.* **35**, 122.
Dunn (1925). *J. Amer. chem. Soc.* **47**, 2564.
—— (1938). The chemistry of the amino-acids and proteins. Springfield, Illinois (ed. Schmidt).
Eck & Marvel (1939). *Organic Synth.* **19**, 61.
Fearon (1939). *Biochem. J.* **33**, 902.
Felix & Mager (1937). *Hoppe-Seyl. Z.* **249**, 111.
Fischer (1903). *Hoppe-Seyl. Z.* **39**, 155.
—— (1907). *Ber. dtsch. chem. Ges.* **40**, 1501.
—— & Jacobs (1906). *Ber. dtsch. chem. Ges.* **39**, 2942.
—— & Skita (1902). *Hoppe-Seyl. Z.* **35**, 221.
Friedemann & Kendall (1929). *J. biol. Chem.* **82**, 23.
Hildebrand (1921). *J. Amer. chem. Soc.* **43**, 500.
Lecat (1930). *J. phys. Chem.* **34**, 2508.
—— (1932). L'Azeotropisme. Brussels.
Ledbury & Blair (1925). *J. chem. Soc.* p. 2832.
Lesesne & Lochte (1938). *Industr. Engng Chem.* Anal. ed. **10**, 450.
Lipmann & Levene (1932). *J. biol. Chem.* **98**, 109.

Macara & Plimmer (1940). *Biochem. J.* **34**, 1431.
McCoy, Meyer & Rose (1935). *J. biol. Chem.* **112**, 283.
Malaprade (1934). *Bull. Soc. chim. Fr.* (5), **1**, 833.
Martin & Synge (1940). *Nature, Lond.*, **146**, 491.
— — —— (1941). *Biochem. J.* **35**, 91.
Matuszak & Frey (1937). *Industr. Engng Chem.* Anal. ed. **9**, 111.
Nicolet & Shinn (1939). *J. Amer. chem. Soc.* **61**, 1615.
Osborne & Jones (1910). *Amer. J. Physiol.* **26**, 212.
Peters & Van Slyke (1932). Quantitative Clinical Chemistry. London.
Posternak & Posternak (1927). *C.R. Acad. Sci., Paris*, **185**, 615.
Schicktanz, Etienne & Steele (1939). *Industr. Engng Chem.* Anal. ed. **11**, 420.
—— Steele & Blaisdell (1940). *Industr. Engng Chem.* Anal. ed. **12**, 320.
Schryver, Buston & Mukherjee (1925). *Proc. roy. Soc.* B, **98**, 58.
Sharp (1939). *Biochem. J.* **33**, 679.
Sørensen (1905). *C.R. Lab. Carlsberg*, **6**, 137.
Synge (1939). *Biochem. J.* **33**, 1924.
Van Slyke & Hiller (1921). *Proc. Nat. Acad. Sci., Wash.*, **7**, 185.
—— Hiller, Dillon & MacFadyen (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 548.
—— —— MacFadyen, Hastings & Klemperer (1940). *J. biol. Chem.* **133**, 287.
Vickery & Schmidt (1931). *Chem. Rev.* **9**, 169.
Vorlander (1929). *Z. anal. Chem.* **77**, 241.
Wada (1933). *Biochem. Z.* **257**, 1.
Walker (1940). *Industr. Engng Chem.* **32**, 1016.
West & Carter (1937). *J. biol. Chem.* **119**, 109.

APPENDIX

X-RAY INVESTIGATION OF THE 2:4-DINITROPHENYLHYDRAZINE COMPOUNDS WITH VARIOUS ALDEHYDES AND KETONES AND WITH PRODUCTS LIBERATED BY PERIODIC ACID FROM PROTEIN HYDROLYSATES

By FLORENCE O. BELL

The 2:4-dinitrophenylhydrazone of the compound obtained from wheat gluten by the action of periodic acid (see above) is a mixture of yellow and orange-red crystals; acetaldehyde 2:4-dinitrophenylhydrazone as usually prepared by crystallization from solvents is yellow, and the propaldehyde derivative (however prepared) is red. The melting point of the gluten derivative is lower than that of the yellow acetaldehyde derivative, and it is therefore possible that the gluten compound is formed from acetaldehyde (derived from threonine) with small amounts of propaldehyde (derived from a higher homologue of threonine). In an attempt to determine the nature of the gluten compound, X-ray powder photographs were taken of the gluten 2:4-dinitrophenylhydrazine derivative, of the hydrazones formed with acetaldehyde, propaldehyde and acetone respectively, and of various mixtures of these derivatives.

Direct comparison photographs of specimens were taken on two halves of one and the same photographic film, the specimens being exactly interchangeable so that specimen-to-film distance, development conditions etc. were the same for both photographs.

Crystals of the gluten aldehyde derivative ground to a powder were compared with the powdered derivatives of acetaldehyde, propaldehyde and acetone respectively, but the numbers, distribution and intensities of the lines in the gluten photograph were not the same as those of the other three compounds, although certain strong lines appeared in approximately the same positions in all four photographs: the gluten derivative was certainly not identical with the yellow crystals of the acetaldehyde derivative, whereas aldehydes derived similarly from casein, gelatin, wool and gliadin were in all cases identified, in the form of the 2:4-dinitrophenylhydrazone, as acetaldehyde. On the assumption, then, that in addition to acetaldehyde another aldehyde or ketone was present in the gluten preparation, the latter was compared with various mixtures of aldehyde and ketone derivatives.

It was noticed at this stage, however, that fused yellow crystals of acetaldehyde 2:4-dinitrophenylhydrazone give on cooling *red* crystals, similar to those of the gluten and propaldehyde derivatives, and that there are pronounced differences between the X-ray photographs (see Pl. 2, figs. 1, 2) of these yellow and red dimorphs. Further, we found that this dimorphism of acetaldehyde 2:4-dinitrophenylhydrazone had already been noted and studied crystallographically and optically by Bryant [1933].

In spite of this promising clue, the X-ray photograph of the gluten compound (also fused in this case) still did not correspond exactly with that of the red metastable acetaldehyde derivative (see Pl. 2, fig. 3), although it had similar intensities and distribution of lines. Small quantities of propaldehyde or acetone added to the acetaldehyde produced marked effects on the X-ray photograph of the 2:4-dinitrophenylhydrazone (see Pl. 2, figs. 4, 5), as would be expected from Bryant's results: he observed that very small amounts of propaldehyde mixed with acetaldehyde in the 2:4-dinitrophenylhydrazone modified the optical constants of the crystals considerably.

It seems clear, therefore, from these investigations that the aldehyde liberated from gluten hydrolysates by means of periodic acid is at any rate not pure acetaldehyde, since the photograph of its 2:4-dinitrophenylhydrazone does not agree strictly either with that of the yellow or with that of the red form of acetaldehyde 2:4-dinitrophenylhydrazone. No artificial mixture of aldehydes or ketones has so far been found which exactly corresponds to the gluten compound. A mixture of three parts of the acetaldehyde derivative and one part of the propaldehyde derivative crystallized out together from alcohol (see Pl. 2, fig. 6), and also a fused mixture of the acetaldehyde derivative with 2% of the propaldehyde derivative, both gave X-ray photographs closely corresponding to that of the gluten compound, crystallized and fused respectively, but again neither was really identical with it. The presence of small quantities of acetone in the gluten compound appears definitely unlikely from the X-ray photographs. There is some contaminant, nevertheless, and moreover a contaminant that predisposes the acetaldehyde derivative to crystallize in the red form just as though it had been prepared from the melt; but what this contaminant is it must be confessed is still an open question. It is tempting to assume that a small proportion of propaldehyde or of some higher aldehyde is present, but the problem is so complicated by dimorphism and mixed crystallization effects that it would require much more time to investigate satisfactorily than is immediately available, so it is better to suspend judgment for the moment.

I am much indebted to Dr K. M. Rudall for taking a number of the X-ray photographs, and to Dr W. T. Astbury for the benefit of valuable discussion.

EXPLANATION OF PLATE 2

Fig 1 Stable yellow acetaldehyde 2 4-dinitrophenylhydrazone.

Fig 2 Comparison of metastable red acetaldehyde 2:4-dinitrophenylhydrazone (left) and propaldehyde 2 4 dinitrophenylhydrazone (right).

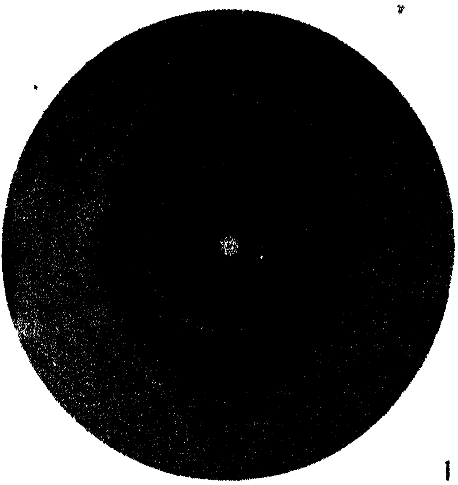
Fig 3 Comparison of red acetaldehyde 2 4-dinitrophenylhydrazone (left) and fused gluten compound 2 4-dinitrophenylhydrazone (right)

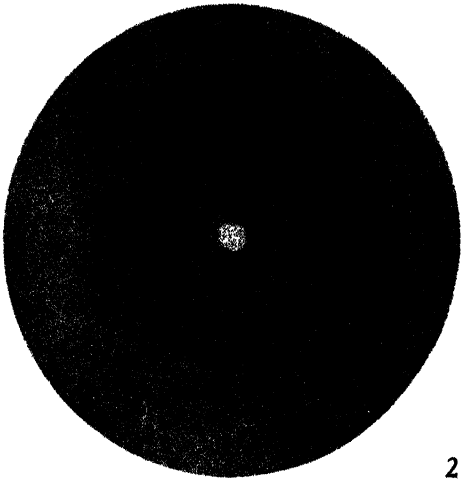
Fig. 4. Comparison of red acetaldehyde 2.4-dinitrophenylhydrazone (left) and acetaldehyde 2:4-dinitrophenylhydrazone plus 2 % propaldehyde 2 4-dinitrophenylhydrazone fused (right)

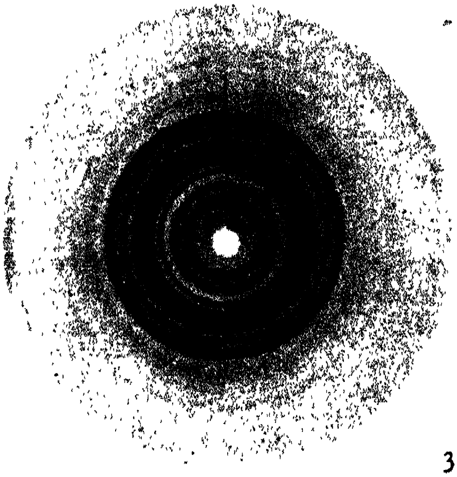
Fig 5. Comparison of red acetaldehyde 2 4-dinitrophenylhydrazone (left) and acetaldehyde 2.4-dinitrophenylhydrazone plus 2 % acetone 2:4-dinitrophenylhydrazone fused (right)

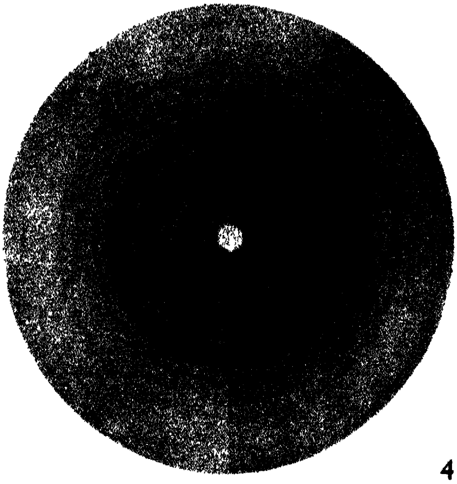
Fig 6. Comparison of gluten compound 2 4 dinitrophenylhydrazone (left) and acetaldehyde 2:4-dinitrophenylhydrazone plus 25 % propaldehyde 2·4-dinitrophenylhydrazone crystals (right)

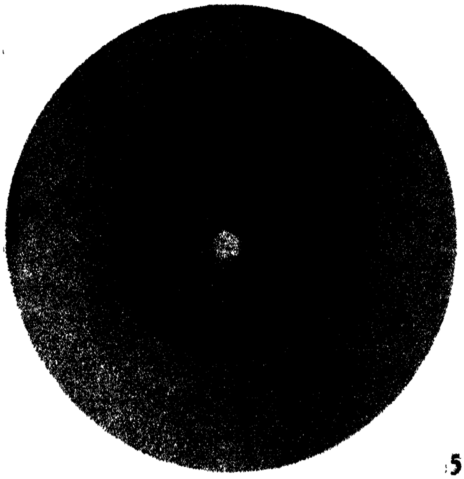
All X-ray photographs taken with Cu K_α radiation, and specimen to film distance approximately 3 cm.

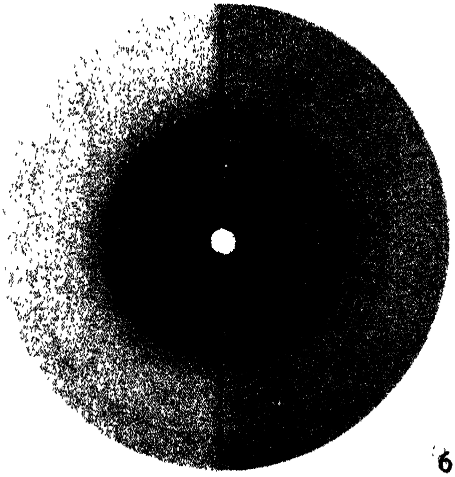

1


2


3


4


5


6

Figs. 1-6.

31. ANALYSIS OF PROTEINS

13. CASEO-PHOSPHOPEPTONE

By J. LOWNDES, T. J. REW MACARA and R. H. A. PLIMMER

*From the Chemistry Department, St Thomas's Hospital
Medical School, London, S.E.*

(*Received 24 February 1941*)

Posternak [1927, 1, 2] first prepared a polypeptide from casein with a P : N ratio of 4 : 15 or 16, and isolated from it the equivalents of 3 mol. glutamic acid, 0·6 mol aspartic acid, 3 mol. *iso*leucine, 3 mol. serine and 4 mol. H_3PO_4. Evidently, on account of decomposition of serine during hydrolysis, he concluded that the phosphopeptone contained 4 mol. phosphoserine. Rimington [1927, 1, 2] isolated a nonapeptide which was considered to consist of 3 mol. hydroxyglutamic acid, 4 mol. hydroxyaminobutyric acid, 2 mol. serine and 3 mol. H_3PO_4. The composition of the phosphopeptone was further examined by Levene & Hill [1933], who obtained from it a dipeptide of glutamic acid and phosphoserine. Previously, Lipmann & Levene [1932] had isolated the Ba salt of phosphoserine from a polypeptide from vitellin and Lipmann [1933, 1, 2, 3] isolated it from a phosphopeptone from casein. Glutamic acid and serine were again isolated by Schmidt [1934]. The presence of glutamic acid in a phosphopeptone from vitellin could not be established by Levene & Schormüller [1933]. Sorimati [1939] considered that he obtained the Ba salt of phosphoserylglutamic acid. The only definite products from caseo-phosphopeptone thus seem to be glutamic acid and serine. On account of the contradictory results of Posternak and Rimington and in connexion with our work on dephosphocasein and dephosphocaseose [Macara & Plimmer, 1940] experiments were carried out on caseo-phosphopeptone. A preliminary account of our results was communicated to the Biochemical Society in March 1940. Caseo-phosphopeptone appeared to be an octapeptide with 2 mol. serine, 2 mol. glutamic acid and 2 mol. H_3PO_4. Rimington [1941] consequently re-examined his caseopeptone; he has consulted with us and kindly shown us his paper before publication. His product is now considered to be a nona- or deca-peptide with 5 mol. glutamic acid, 1 mol. *iso*leucine, 4 mol. serine and 3 mol. H_3PO_4. Owing to unforeseen circumstances our work had to cease, but we feel that our data, so different from those of Rimington, should appear simultaneously. Damodaran & Ramachandran [1941] have given the composition of caseopeptone as a decapeptide with 3 mol. glutamic acid, 4 mol. serine, 3 mol. *iso*leucine and 3 mol. H_3PO_4. There is thus some general agreement about the composition of the phosphopeptone, but the number of mol. of each amino-acid needs further examination.

EXPERIMENTAL

Preparation of caseo-phosphopeptone

Crude Na-phosphopeptone was prepared from 'Rhenania' casein, purchased by one of us many years ago, according to the method of Levene & Hill [1933], using portions of 50 g. casein and 1·5 g. trypsin (B.D.H.). The casein contained

10·1 % H_2O, 13·44 % N and 0·82 % P. The average yield was 3–4 g. or 6–8 %, with a N : P ratio of 4·5–5 : 1. Na_2CO_3 was invariably present.

For purification, several crops were combined, dissolved in water, the Na_2CO_3 neutralized with acetic acid, the Pb salt again precipitated and converted back into the Na salt. This was carried out three times and the Na salt thrown down with alcohol. The alcohol decanted from the syrupy precipitate contained only traces of N and P, and the syrup had a consistent N : P ratio of 4 : 1. The free acid was then made by again precipitating the Pb salt, which was washed and decomposed with H_2S. The filtrate from PbS was concentrated *in vacuo* to a small volume, filtered from the last traces of PbS, further concentrated, transferred to a basin, treated with alcohol and acetone, dried *in vacuo*, and ground to a fine powder. The yield of purified phosphopeptone was approx. 4 %. Its P content corresponded with approx. 25 % of the original casein. Inorganic phosphate liberated by trypsin corresponded with 11–12 %. The remaining 63 % not precipitated by lead acetate or alcohol awaits further investigation.

Dried *in vacuo* over $CaCl_2$, the purified caseo-phosphopeptone contained 10·56 % N and 5·77 % P. Hence N : P = 4 : 1. Amino-N determination showed 12·6 % of the total N. The phosphopeptone thus appears to be an octapeptide with 2 atoms of P.

Sulphur and diamino-acids are absent, tyrosine and tryptophan reactions are negative. Hydrolysis with 20 % HCl for 24 hr. raised the amino-N to 88 % of the total N, for 36 hr. to 98 %, and for 48 hr. to 100 %, which suggests a more resistant peptide linkage in the molecule, and shows the absence of cyclic N.

The solution of phosphopeptone is very acid in reaction:

0·2704 g. = 13·9 ml. *N*/10 NaOH to either phenol- or thymol-phthalein. On then adding neutralized formalin, the figure was raised to 15·7 ml. 0·2704 g. = 28·35 mg. N is thus equivalent to 1·39 mg. H and after formalin to 1·57 mg. H.

Ratio of acidic H to N atoms = 1·39 : 28·35/14 = 0·686/1 or 5·5/8
= 1·57 : 28·35/14 = 0·775/1 or 6·2/8.

The octapeptide would thus have six replaceable H atoms. Two will belong to the two H_3PO_4 radicles, two to 2 mol. glutamic acid (see below), one to a COOH group at the end of the chain, and the sixth to another COOH group.

Liberation of P by N/4 NaOH at 37°. For comparison with caseinogen which loses all its P as PO_4 ions in 24 hr. at 37°, 0·4240 g. phosphopeptone was dissolved in 100 ml. *N*/4 NaOH and placed in an incubator at 37°. Samples were taken at intervals and inorganic PO_4 precipitated as NH_4MgPO_4 and estimated by our usual procedure:

At 0 hr.	inorg. PO_4	=0·421	mg. P	=17·9	% hydrolysis.
4	„	=0·570	„	=24·3	„
8	„	=1·566	„	=66·7	„
12	„	=1·810	„	=77·1	„
24	„	=2·040	„	=87·0	„
48	„	=2·048	„	=87·3	„
	Total P	=2·346			

Rather more than half the P is separated in 8 hr. and the hydrolysis is not complete in 24 hr. and not increased in 48 hr. The difference in rate may be due to the position of the phosphoserine in the chain, one more easily hydrolysed in the middle portion, the other at the end of the chain.

The results contrast with the stability of phosphoserine (later publication) which is not hydrolysed by *N*/4 NaOH at 37° and at 100° loses 66 % in 48 hr.

Separation of H_3PO_4 by hydrolysis with acid. For future work on the isolation of amino-acids and confirmation of the production of a dipeptide of glutamic acid and phosphoserine hydrolysis by HCl at 100° was investigated:

With 5·5 *N* HCl for 24 hr. inorg. PO_4 separated was 53·85 % of total P
5·5 „ 48 „ „ 100 „
2 „ 10 „ „ 15·4 „

In comparison with phosphoserine which lost 60 % of its P as inorg. PO_4 by boiling with *N* HCl for 24 hr. the phosphopeptone is more stable.

Estimation of glutamic acid. The opportunity was taken of estimating glutamic acid by the micro-enzymic method of Cohen [1939] at the same time as in dephosphocasein and dephosphocaseose [Macara & Plimmer, 1940].

1 g. phosphopeptone was hydrolysed with 5·5 *N* HCl for 24 hr. The solution was evaporated *in vacuo* several times to remove HCl, the resulting syrup dissolved in water, neutralized to *p*H 3 and adjusted to 50 ml. A nitrogen estimation showed the concentration to be 2 %. The procedure as described by Macara & Plimmer was then carried out:

4 ml. hydrolysate contained 22·1 mg. glutamic acid − 27·6 %
5 „ „ 25·9 „ = 25·9 %
Mean 26·8 %

The mean value is 24·2 % of the total N, which indicates the presence of 1 mol. per 4 atoms of N or 2 mol. in 1 mol. of octapeptide.

Isolation of glutamic acid. 5 g. phosphopeptone were hydrolysed with 50 ml. 5·5 *N* HCl. The solution was concentrated *in vacuo* and saturated with dry HCl gas. Glutamic acid hydrochloride slowly crystallized out. Dried *in vacuo* the crude salt weighed 0·8 g. = 16 % of the phosphopeptone. The yield is far below the figure by estimation. Precipitation is probably inhibited by the presence of serine and its decomposition products.

Estimation of threonine. To establish the presence and determine the amount of this amino-acid the method of Block & Bolling [1939] as modified by Macara & Plimmer [1940] was used.

1·3824 g. phosphopeptone were hydrolysed with 5·5 *N* HCl for 36 hr. and the volume adjusted to 100 ml.; 2 ml. of this solution after treatment gave only a mere trace of colour with the reagent.

It had to be concluded that threonine was absent from the octapeptide.

Estimation of total β-hydroxyamino-acids. According to Van Slyke *et al.* [1940] oxidation of β-hydroxyamino-acids with KIO_4 completely separates the amino group as NH_3. The same hydrolysate was used as for threonine.

5 ml. gave 7·3 ml. *N*/14 NH_3 = 10·56 % total N
5 ml. gave 1·1 ml. *N*/14 NH_3 by aeration with Na_2CO_3 = 15 % of total N
and 1·2 ml. *N*/14 NH_3 on oxidation with KIO_4 = 16 % of total N

Assuming that amide groups are not present in the phosphopeptone and that the NH_3 liberated by Na_2CO_3 arises from decomposition of serine by boiling with HCl (see below) the total of 31 % of the total N indicates the presence of 2 mol. serine in the octapeptide if hydroxyglutamic is not present. Harington & Randall [1931] could not isolate this compound from caseinogen and Gulland & Morris [1934] obtained a doubtful 0·33 %.

Estimation of serine. Attempts to estimate serine by Rapoport's method [1937] were not successful; only brown charred solutions resulted [cf. Macara & Plimmer, 1940].

Liberation of NH_3 by hydrolysis. Posternak [1927, 1] noted the liberation of NH_3 during acid hydrolysis of phosphopeptone and regarded it as indicative of serine peptides and later [1928] found 12 % evolved from caseopeptone and 20 % from vitellopeptone. As higher amounts were evolved with greater concentrations of acid he considered it did not arise from amide groups. Serine under the same conditions gave 8 %. Hydrolysis of caseopeptone with NaOH gave higher amounts. Further examination of the liberation of NH_3 seemed advisable with serine, phosphoserine and caseopeptone.

Serine. (*a*) 0·5 g. serine was boiled under reflux with 60 ml. 5·5*N* HCl. Samples were taken every 12 hr., made alkaline with Na_2CO_3 and the NH_3 aerated into standard acid.

(*b*) A similar experiment was made with the addition of 1 equiv. of H_3PO_4 to the solution.

	With HCl only		With H_3PO_4	
After 12 hr.	NH_3 = 0·294 mg. N	= 2·7 % total N	= 0·294 mg. N	= 2·7 % total N
24 ,,	= 0·399 ,,	4·0 ,,	= 0·370 ,,	= 3·3 ,,
36 ,,	= 0·588 ,,	= 5·3 ,,	= 0·955 ,,	8·6 ,,
60 ,,	= 0·785 ,,	= 6·7 ,,	= 1·920 ,,	= 17·1 ,,

(*c*) Plimmer & Lowndes [1938] found 17·9 % N liberated on boiling serine for 6 hr. with 5*N* NaOH.

Phosphoserine. 0·11 g. was dissolved in 100 ml. 2*N* NaOH. Total N in 10 ml. = 0·7 mg. The remaining 90 ml. (= 6·3 mg.) diluted with 90 ml. H_2O were distilled and the NH_3 collected at 15 min. intervals in standard acid:

In 15 min. NH_3 evolved = 0·07 mg.
In next 15 min. NH_3 evolved = 0·07 mg.
,, ,, = 0·07 mg.
After 75 min. total = 0·30 mg. = 4·76 % of total N

Caseopeptone. (*a*) As stated above under KIO_4 oxidation 15 % of the total N was evolved as NH_3 on boiling with 5·5*N* HCl for 36 hr.

(*b*) 0·2694 g. was dissolved in 200 ml. *N* NaOH and distilled for 1 hr. The NH_3 evolved was 21·3 % of the total N.

(*c*) 0·3 g. was dissolved in 100 ml. 2*N* NaOH. 10 ml. were taken for total N = 3·55 mg. The remaining 90 ml. were diluted with 90 ml. H_2O and distilled and the NH_3 collected in standard acid and titrated at 15 min. intervals. The volume was kept constant by occasional addition of water.

After 15 min.	NH_3 = 5·00 mg. N	= 15·7 % of total N (31·95 mg.)
30 ,,	= 6·02 ,,	= 18·8 ,, ,,
45 ,,	= 6·53 ,,	= 20·4 ,, ,,
60 ,,	= 7·04 ,,	= 22·0 ,, ,,
75 ,,	= 7·40 ,,	= 23·2 ,, ,,
90 ,,	= 7·62 ,,	= 23·9 ,, ,,
105 ,,	= 7·84 ,,	= 24·5 ,, ,,
120 ,,	= 8·13 ,,	= 25·5 ,, ,,

Serine and phosphoserine are comparatively stable to hydrolysis by acid and alkali. Caseopeptone is unstable to both. On plotting the figures of NH_3 liberation by alkali on a curve, the rapid evolution in the first 15 min. is equivalent to 1 atom of N out of the 8 atoms in the octapeptide (12·5 % of total N). A slower liberation follows up to 25·5 % in 120 min., which is equivalent to 2 atoms of N of the octapeptide. The difference in rate suggests loss of NH_3 from phosphoserine groups at the end or middle of the chain, a free amino group at the end being very labile.

SUMMARY AND DISCUSSION

Our preparation of caseo-phosphopeptone, judging from its N and amino-N contents, is an octapeptide containing two H_3PO_4 groups, 2 mol. glutamic acid and 2 mol. serine. Its acidity indicates the presence of another mol. of a dicarboxylic acid, leaving 3 mol. of simple amino-acids.

As laboratory work has been interrupted, calculations of molecular weight and deductions therefrom may be allowed:

From P content of 5·756 % and 2 atoms per mol.: mol. wt. is $\frac{2 \times 31 \times 100}{5{\cdot}765} = 1079$

From glutamic acid content of 26·8 % and 2 mol.: mol. wt. is $\frac{2 \times 147 \times 100}{26{\cdot}8} = 1097$

From NH_2-N figure of 1 33 %: mol. wt. is $\frac{14 \times 100}{1{\cdot}33} = 1053$

Mean = 1076

Deduct 2 mol. glutamic acid 2 × 147	= 294	
2 mol. serine 2 × 105	= 210	
2 mol. H_3PO_4 2 × 98	= 196	
	700	
Less 2 ester linkages and 7 peptide linkages 9 × 18	= 162	
	538	538
Mol. wt. of remaining 4 mol.		= 538
Mean mol. wt. of amino-acids	= 134	
Mol. wt. of leucines	= 131	
Mol. wt. of aspartic acid	= 134	

The deductions are confirmatory of the isolation of 3 mol. of *iso*leucine by Posternak, and by Damodaran & Ramachandran. The latter authors find caseo-phosphopeptone to be a decapeptide with 3 mol. glutamic acid, 3 or 4 mol. serine and 3 mol. H_3PO_4. Rimington's phosphopeptone is a nona- or decapeptide. The isolation of a dipeptide of glutamic acid and phosphoserine by Levene & Hill points to the combination of these two amino-acids. In view of the rapid liberation of 1 mol. NH_3 by the action of $N/4$ NaOH and a difference in the rate of separation of H_3PO_4 we suggest that one phosphoserylglutamic group is at the end of the chain with a free amino group easily liberated, and the second group is in the middle of the chain, and picture the molecule of the octapeptide thus: phosphoserylglutamic-X-X-phosphoserylglutamic-X-X, where X is the unknown amino-acid, probably 3 mol. *iso*leucine and 1 mol. aspartic acid.

The difference between our octapeptide and the decapeptide seems to be 1 mol. of dipeptide of serine and glutamic acid, possibly at the other end of the chain from which it may be more easily split off: phosphoserylglutamic-X-X-phosphoserylglutamic-X-X-phosphoserylglutamic.

There is no evidence whether the dipeptide is phosphoserylglutamic acid, or glutamylphosphoserine. Both isomers may be present, but the one at the left-hand end seems to be phosphoserylglutamic acid. Phosphoserine may also be in combination with *iso*leucine or other amino-acid, if present.

REFERENCES

Block & Bolling (1939). *J. biol. Chem.* **130**, 365.

Cohen (1939). *Biochem. J.* **33**, 551.

Damodaran & Ramachandran (1941). *Biochem. J.* **35**, 122.

Gulland & Morris (1934). *J. chem. Soc.* p. 1644.

Harington & Randall (1931). *Biochem. J.* **25**, 1917.

Levene & Hill (1933). *J. biol. Chem.* **101**, 711.

—— & Schormüller (1933). *J. biol. Chem.* **103**, 537.

Lipmann (1933, 1). *Naturwissenschaften*, **21**, 236.

—— (1933, 2). *Biochem. Z.* **262**, 3.

—— (1933, 3). *Biochem. Z.* **262**, 9.

—— & Levene (1932). *J. biol. Chem.* **98**, 109.

Macara & Plimmer (1940). *Biochem. J.* **34**, 1431.

Plimmer & Lowndes (1938). *C.R. Lab. Carlsberg*, **22**, 434.

Posternak (1927, 1). *C.R. Acad. Sci.* **184**, 306.

—— (1927, 2). *Biochem. J.* **21**, 289.

—— (1928). *C.R. Acad. Sci.* **187**, 313.

Rapoport (1937). *Biochem. Z.* **289**, 400.

Rimington (1927, 1). *Biochem. J.* **21**, 1179.

—— (1927, 2). *Biochem. J.* **21**, 1187.

—— (1941). *Biochem. J.* **35**, 321.

Schmidt (1934). *Hoppe-Seyl. Z.* **223**, 86.

Sorimati (1939). *J. Biochem., Tokyo*, **29**, 289.

Van Slyke, Hiller, MacFadyen, Hastings & Klemperer (1940). *J. biol. Chem.* **133**, 287.

32. NOTE ON THE AMINO-ACIDS PRESENT IN PHOSPHOPEPTONE

By CLAUDE RIMINGTON

From the National Institute for Medical Research, London, N.W.3

(*Received 26 February 1941*)

Some years ago Rimington & Kay [1926] gave the name 'phosphopeptone' to a substance present in tryptic digests of caseinogen and later isolated [Rimington, 1927, 1] by a process involving separation of inert material and then precipitation of the phosphorus-rich complex as an insoluble uranyl derivative and lastly as a copper salt.

The N/P ratio of the crude copper salt was approximately 3·3/1, but by fractional copper precipitation a main homogeneous constituent with N/P = 3·0 was obtained and used for detailed study of amino-acid composition, etc. The fractions with higher and lower N/P ratios were not analysed in this way, but it was realized that peptones of varying composition might easily be obtained as a result of more or less prolonged tryptic digestion before isolation was commenced. The main phosphopeptone was not completely stable towards activated pancreatic juice [Rimington & Kay, 1926].

Examination of phosphopeptone by the methods then available for the identification of amino-acids [Rimington, 1927, 2] revealed the presence of serine and a dicarboxylic acid considered to be hydroxyglutamic acid and also an incompletely identified substance thought, possibly, to be β-hydroxy-α-amino-butyric acid.

Since these early studies, investigations have been published by several workers. Schmidt [1934], after prolonged tryptic digestion of caseinogen, isolated a substance with N/P = 2, composed of glutamic acid and serine; fractions with higher N/P ratios were also encountered, when the digestion was less complete, in which a leucine was also present. Levene & Hill [1933], combining a preliminary tryptic digestion of caseinogen with subsequent hydrolysis by boiling acid, succeeded in isolating from the mixture a dipeptide of glutamic acid, serine and phosphoric acid, whilst Lipmann [1933] by boiling acid alone obtained from his hydrolysates serine phosphoric acid; peptides of higher N/P ratio were also present in the mixtures. More recently Damodaran & Ramachandran [1940], in a preliminary communication, claimed to have isolated a phosphorus-containing peptone from caseinogen by applying pepsin, separating the paranuclein formed and then digesting with trypsin. It had N/P = 3·3 and is reported to contain 3 mol. glutamic acid, 3 mol. *iso*leucine and 4 mol. serine.

Although it is only to be expected that the use of different chemical or enzymic hydrolyses (and for different lengths of time) should lead to phosphorus-containing materials of different constitution, it was thought desirable to re-examine the original 'phosphopeptone' preparations, now that more reliable methods are available for the detection and determination of amino-acids like threonine. The isolation of serine phosphoric acid after acid hydrolysis of caseinogen by Levene & Hill [1933] supports my conclusion that a part, at least, of the phosphorus in phosphopeptone is united to serine, but from the colorimetric determinations presented by Rapoport [1937] the question arises whether or not all the phosphorus in caseinogen is bound in this way.

EXPERIMENTAL

There was available for this work a small quantity of the original phosphopeptone described in 1927 and a larger quantity of crude copper salt prepared subsequently. From the latter the copper was removed as sulphide, employing fuller's earth to facilitate filtration [Rimington, 1927, 1], and the free peptone was precipitated by addition of excess of alcohol. After dehydration with absolute alcohol and ether it was dried *in vacuo* at 105° over P_2O_5. Both peptones were colourless powders, readily soluble in water to form strongly acid solutions.

Analysis. Determination of N/P ratios gave the values 3·01/1 and 3·33/1 for the original peptone and the larger sample respectively. Since amino-N determinations and other data indicate that there are 3 P atoms per mol., these substances contained 9 and 10 N atoms respectively and will be referred to as the N_9 and N_{10} peptones. Elementary analysis gave the following results:

	C	H	N	P
N_9 peptone	35·29	6·16	9·86	7·27
$C_{37}H_{60}O_{33}N_9P_3$ requires	35·50	4·80	10·07	7·44
N_{10} peptone	37·58	6·16	10·40	6·88
$C_{43}H_{71}O_{34}N_{10}P_3$ requires	37·83	5·21	10·27	6·82

It will be noticed that the empirical formulae, deduced from the analytical figures and assuming 3 phosphoric acid residues per mol., differ by one molecule of leucine or *iso*leucine, $C_6H_{13}O_2N$, in peptide linkage.

Determination of dicarboxylic acids and isolation of glutamic acid

Hydrolysates of the N_{10} and N_9 peptones were prepared by boiling for 48 hr. with 20 % HCl. Portions of these were worked up by Foreman's quantitative calcium salt method for the determination of dicarboxylic amino-acids. The result in each case corresponded to rather less than 4 mol. of dicarboxylic acid per mol. of peptone, thus:

N_9 peptone:	total N in hydrolysate	19·72 mg.
	4/9 of total N	8·76 „
	total N in Ca salt fraction	8·64 „
N_{10} peptone:	total N in hydrolysate	166·0 „
	4/10 of total N	66·4 „
	total N in Ca salt fraction	59·3 „

Since hydroxyglutamic acid had previously been suspected, in the dicarboxylic acid fraction, calcium was removed from a portion by means of oxalic acid and the crude amino-acid obtained oxidized with chloramine T as described by Harington & Randall [1931]. The mixture was treated with *p*-nitrophenylhydrazine and warmed on the water bath for 45 min., then filtered and the residue washed repeatedly with boiling methyl alcohol until a small dark red residue remained, a trace of which afforded an intense pure blue colour with alcoholic KOH. The substance was recrystallized from hot nitrobenzene, from which it separated in the form of dark red needles melting at 322–7°. The yield was only 4·4 mg. from 0·3 g. of starting material and the substance contained 22·6 % N. It was considered to be the *p*-nitrophenylosazone of glyoxal, since the M.P. was not lowered by admixture with this substance (N = 25·6 %). Mixed with the corresponding derivative of malic semi-aldehyde (N = 21·7 %), kindly supplied by Prof. Harington, the M.P. was depressed approximately 20°. Unfortunately the M.P. of these substances are not very suitable for their characterization; however, since serine is known to be unstable under the hydrolytic conditions employed and could readily yield glyoxal, the conclusion is probably justified that serine was present as an impurity in the crude dicarboxylic acid fraction examined.

Further evidence against the presence of any α-amino-β-hydroxy-acid in this fraction was obtained by treating an aliquot portion with periodic acid [Van Slyke *et al.* 1940], which failed to liberate any ammonia.

The remainder of the crude dicarboxylic fraction was freed from calcium by oxalic acid and concentrated to a small volume. Crystals separated which were filtered off, washed sparingly and dried (crop 1) and, by further concentration of the mother liquors, a second deposit (crop 2) was obtained. The final mother liquors were treated with several volumes of alcohol, when a small amount of crystalline material (crop 3) slowly separated. These fractions were analysed with the following result:

	C	H	N
Crop 1 (M.P. 198°)	41·01	6·05	9·64
Crop 2	40·59	6·28	9·50
Crop 3 (corrected for ash)	36·45	6·49	—
Glutamic acid requires	40·85	6·17	9·52
Hydroxyglutamic acid requires	36·81	5·52	8·59

It would appear, in the light of the experiments already recorded, that crop 3 consisted only of impure glutamic acid. There was unfortunately insufficient material for N determination. In view of the similar C and H figures which were previously reported [Rimington, 1927, 2] for the dicarboxylic acid fraction of phosphopeptone, it is noteworthy that impure glutamic acid may give analytical results so closely approaching those required by hydroxyglutamic acid.[1]

The optical rotatory power of the material of crop 1 in 10 % HCl was $[\alpha]_{5461} = +37{\cdot}39^\circ$. The hydrochloride was easily prepared in 88 % yield and, dissolved in water (3·2 %), had $[\alpha]_D = +22{\cdot}04^\circ$, $[\alpha]_{5461} = +27{\cdot}11^\circ$. Anslow & King [1927] give $[\alpha]_{5461}$ as 26·85° for a 2·5 % aqueous solution and 27·45° for a 5 % solution.

Past attempts to isolate glutamic acid as the hydrochloride directly from phosphopeptone hydrolysates had not succeeded and the separation has now only been achieved with difficulty and in poor yield. It would appear that the other products of hydrolysis, such as serine and phosphoric acid, exert an inhibiting effect upon the crystallization of the hydrochloride.

5 g. of N_{10} peptone were refluxed with 50 ml. of 20 % HCl for 30 hr. and the mixture then saturated with HCl gas. In spite of seeding, not more than a trace of material separated after 24 hr. at 0°. The liquid was therefore concentrated to small volume and again saturated with HCl, when 1·4 g. of crystals separated in the course of several days. Repeated concentration and saturation raised the yield to 1·48 g., which represents only between 2 and 3 mol. per mol. of peptone.

It must be concluded from the above findings that glutamic acid and not hydroxyglutamic acid is present in phosphopeptone.

Isolation and identification of isoleucine *from* N_{10} *peptone*

The filtrate from the calcium salts of a hydrolysate corresponding to 1·6 g. of N_{10} peptone was freed from calcium by oxalic acid, made slightly alkaline to litmus with ammonia and concentrated to about 2·5 ml. Upon cooling in the ice-chest, crystals separated which, after recrystallization from 75 % alcohol, formed fragile glistening plates. Concentration of the mother liquor yielded a further small crop: total yield 73·5 mg. 1 mol. per mol. of peptone corresponds to 155 mg.

---

[1] Prof. A. C. Chibnall informs me that he has often obtained analytical figures for impure glutamic and aspartic acids which correspond closely to those required for hydroxyglutamic acid.

	C	H	N
Analysis: Found	54·88	9·98	10·73
*iso*Leucine ($C_6H_{13}O_2N$) requires	54·97	9·99	10·69

A 1·6 % solution in 20 % HCl had $[\alpha]_D = +34·38°$; $[\alpha]_{5461} = +40·62°$. The $[\alpha]_D$ of *iso*leucine in this solvent is +34·26° and that of leucine is +15·4°. For further identification, the phenylisocyanate derivative was prepared. It had M.P. 117°; that of *iso*leucine melts at 119°.

Examination for threonine

Block & Bolling [1939] have recently described a method for the micro-determination of threonine depending upon its oxidation with lead tetraacetate to acetaldehyde which is determined colorimetrically after reaction with *p*-hydroxydiphenyl; serine does not interfere. When this method was applied to a phosphopeptone hydrolysate only a trace of colour resulted, corresponding to less than 1/40 mol. threonine per mol. of peptone. It is therefore concluded that threonine is absent from phosphopeptone.

Determination of serine and total hydroxyamino-acids

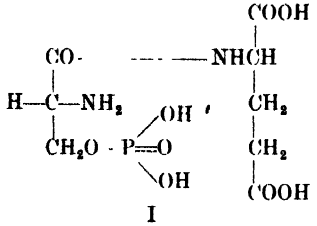
Phosphoserine is remarkably resistant to hydrolysis with boiling acids (personal communication from Prof. R. H. A. Plimmer); this property evidently made possible the isolation of phosphoserine by Lipmann [1933] and by Schmidt [1934] from acid hydrolysates of caseinogen. Serine itself, however, is readily transformed by hot mineral acids into ammonia, glyceric acid, glyoxal and other products (compare Damodaran & Ramachandran [1940]), so that one is beset with the difficulty, in attempting its determination in phosphopeptone, of achieving complete liberation from phosphoric acid with the minimum of destruction. The use of alkali for this purpose was not satisfactory.

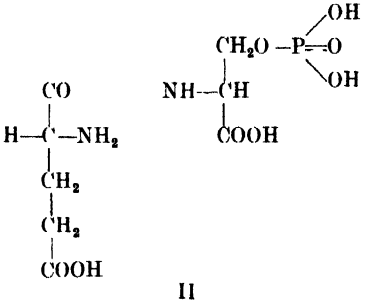
2 g. of N_{10} peptone were refluxed with 20 % HCl for 160 hr., by which time all the phosphorus was present as free phosphoric acid. Direct determination of ammonia by an aeration method showed 26·5 % of the total N to be present in this form. A periodate oxidation liberated another 13·6 %, making in all 40·1 % of the total N, or 4 mol. per mol. of peptone, derivable from hydroxy-amino-acids. On a portion of the hydrolysate, serine was determined by Rapoport's [1937] colorimetric method. The amount present corresponded to only 5·6 % of the total N, in contrast to the figure of 13·6 % of hydroxyamino-acid by periodate oxidation recorded above. This finding is similar to Rapoport's own results with casein hydrolysates, from which he concluded that at least one-third of the total phosphorus of the protein was bound to hydroxyamino-acids other than serine. In view of the known instability of serine under the conditions of hydrolysis it is doubtful, however, if much emphasis should be laid upon such negative data. It is possible that a portion of the serine originally present has been so changed that it is no longer capable of yielding glyceric acid. In fact, Damodaran & Ramachandran [1940] report that they found both lactic and pyruvic acids, presumably derived from serine, in their phosphopeptone hydrolysates.

Nature of the phosphorus linkage in phosphopeptone

The suggestion was made by Rimington [1927, 2] that two of the three phosphoric acid residues of phosphopeptone were held in mono-ester linkage whilst the third was in the form of a di-ester. This suggestion was based upon the observed difference in action of bone and kidney phosphatases upon phosphopeptone, the former liberating only two-thirds of the phosphorus as free phosphoric acid, the latter producing complete hydrolysis. Since phosphoserine is relatively resistant to hydrolysis by boiling dilute alkali, whereas the phosphorus is rapidly split off as phosphoric acid from caseinogen by 1 % NaOH at 37°, another explanation appears possible. The action of alkali upon phosphopeptone was therefore studied again in detail.

It was found that only about two-thirds of the total phosphorus was removed as phosphoric acid by 1 % NaOH at 37° and the time curve of the hydrolysis bore a strong resemblance to that recording the action of bone phosphatase upon phosphopeptone. It appears that the relative stability towards alkali of phosphoserine, or of a terminal phosphoserine residue in peptone linkage with other amino-acids as in phosphopeptone, may be conditioned by the close proximity of the free amino group. Phosphocholine, which as Beznák & Chain [1937] point out, has a betaine structure, was similarly found by them to be remarkably stable towards boiling acids and alkalis although hydrolysed by both bone and kidney phosphatases. Plimmer & Burch [1937] reported the same behaviour on the part of phosphoaminoethanol and phosphocholine. It would be very interesting in this connexion to discover whether Levene & Hill's [1933] phosphodipeptide possessed structure I or II (Levene & Hill have formulated their substance as an aspartic acid derivative).


I


II

Discussion

On the assumption that only glutamic acid, *iso*leucine, serine and phosphoric acid are present in the N_{10} phosphopeptone, it is found possible to construct the following formulae, which are in good agreement with the elementary analysis of the peptone and roughly correspond with the yields of each amino-acid experimentally determined.

N_{10} peptone:

5 glutamic acid + 1 *iso*leucine + 4 serine + $3H_3PO_4 - 12H_2O = C_{43}H_{71}O_{34}N_{10}P_3$,

requiring 37·83 % C; 5·21 % H; 10·27 % N; 6·82 % P.
Found 37·58 % C; 6·16 % H; 10·40 % N; 6·88 % P.

Experimentally not more than 4 mol. of glutamic acid were found by the Ca salt method, but there were also found 4 mol. of serine and at least 1 mol. of *iso*leucine. Similarly for the

N_9 peptone:

5 glutamic acid + 4 serine + $3H_3PO_4 - 11H_2O = C_{37}H_{60}O_{33}N_9P_3$,

requiring 35·50 % C; 4·80 % H; 10·07 % N; 7·44 % P.
Found 35·38 % C; 5·14 % H; 10·03 % N; 7·41 % P.

Once more the glutamic acid determination fell short of the required figure, but the difference in elementary composition between the N_{10} and N_9 peptones corresponds to exactly 1 mol. of *iso*leucine, and it will be remembered that this amino-acid was not detected in the course of the original study made of the N_9 phosphopeptone [Rimington, 1927, 2].

Whilst, then, some reserve must still be exercised, it is felt that the compositions suggested above do probably represent the true nature of the two peptones which have been here subjected to study. Naturally, other conditions of preparation of the caseinogen hydrolysate, e.g. prolonged tryptic digestion, might lead to peptones with a different composition, as the work of Levene & Hill and others clearly indicates; most drastic hydrolysis leads to the comparatively stable phosphoserine.

Damodaran & Ramachandran [1940] considered the phosphopeptone which they prepared to be composed of 3 mol. of glutamic acid, 3 mol. of *iso*leucine, 4 mol. of serine and 3 mol. of phosphoric acid. The yields of these substances which they obtained were in fairly good agreement with such a conclusion, but no elementary analysis of the complete peptone is recorded. This is unfortunate, since elementary analyses would have afforded a check upon the conclusions arrived at. The composition demanded by such an assembly of amino-acids differs considerably from that determined experimentally for my N_{10} peptone, thus:

N_{10} peptone:	C	H	N	P
Found	37·78	5·30	10·25	6·81
Calculated formula $C_{43}H_{78}O_{34}N_{10}P_3$ requires	37·83	5·21	10·27	6·82
Damodaran & Ramachandran's peptone would require	40·54	5·93	10·51	6·98

One must conclude that Damodaran & Ramachandran's peptone, prepared as it is from paranuclein, differs from the phosphopeptone prepared according to my method by the action of trypsin only upon caseinogen. It appears that the essential phosphorus linkage in caseinogen is between phosphoric acid and serine and that this amino-acid is united in peptide linkage with other amino-acids, predominantly glutamic acid but possibly also *iso*leucine, and that tryptic digestion rapidly sets free these complexes from the rest of the protein molecule (compare the evidence of Rimington & Kay [1926]), but that they themselves being more resistant towards enzymic hydrolysis are only slowly degraded into peptones or peptides of ever decreasing complexity. A phosphopeptone unit containing 10 amino-acids and 3 mol. of phosphoric acid is comparatively readily isolated.

This reinvestigation of phosphopeptone is a result of communications made to the Biochemical Society (*Chem. and Ind.* **59**, 185, 1940) by Prof. R. H. A. Plimmer on 'Phosphoryl hydroxyamino-acids' and by Dr J. Lowndes on 'The composition of phosphopeptone'. I have enjoyed the benefit of frequent discussion with the authors and this paper is published with their approval. I desire to thank Prof. Plimmer for a specimen of *dl*-threonine and Prof. Robison for a specimen of the Ba salt of 3-phosphoglyceric acid.

REFERENCES

Anslow & King (1927). *Biochem. J.* **21**, 1168.
Beznák & Chain (1937). *Quart. J. exp. Physiol.* **26**, 201.
Block & Bolling (1939). *J. biol. Chem.* **130**, 365.
Damodaran & Ramachandran (1940). *Nature, Lond.*, **145**, 857.
Harington & Randall (1931). *Biochem. J.* **25**, 1917.
Levene & Hill (1933). *J. biol. Chem.* **101**, 711.
Lipmann (1933). *Biochem. Z.* **262**, 3.
Plimmer & Burch (1937). *Biochem. J.* **31**, 398.
Rapoport (1937). *Biochem. Z.* **289**, 420.
Rimington (1927, 1). *Biochem. J.* **21**, 1179.
—— (1927, 2). *Biochem. J.* **21**, 1187.
—— & Kay (1926). *Biochem. J.* **20**, 777.
Schmidt (1934). *Hoppe-Seyl. Z.* **223**, 86.
Van Slyke, Hiller, MacFadyen, Hastings & Klemperer (1940). *J. biol. Chem.* **133**, 287.

33. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN

2. FORMATION AND PROPERTIES OF CHOLEGLOBIN[1]

By R. LEMBERG, J. W. LEGGE and W. H. LOCKWOOD

From the Institute of Medical Research, Royal North Shore Hospital, Sydney

(*Received 3 March 1941*)

In a preliminary publication [Lemberg, Legge & Lockwood, 1938] we have reported that by the coupled oxidation of haemoglobin and ascorbic acid the prosthetic group of haemoglobin is oxidized. Under appropriate conditions a product, still mixed with haemoglobin, can be obtained, in which the new prosthetic group is combined with native globin. Acids easily split off iron from the product of oxidation and form bile pigments. These observations suggest that the compound is a bile pigment-iron-native globin complex, which we have called choleglobin.

In Part 1 of this series [Lemberg, Legge & Lockwood, 1939] we have discussed the initial mechanism of the reaction. In the present paper we describe in detail the preparation and properties of choleglobin, and we compare it with related substances which also contain easily detachable iron and yield bile pigment on acid splitting. Such substances are verdohaemochromogen obtained from pyridine-haemochromogen by coupled oxidation with ascorbic acid [Lemberg, Cortis-Jones & Norrie, 1938], and the 'pseudohaemoglobin' obtained by Barkan & Schales [1937; 1938] by the action of hydrogen peroxide on haemoglobin in the presence of cyanide.

Experimental

Preparation of choleglobin. We have used solutions of crystalline horse oxyhaemoglobin prepared by the method of Heidelberger [1922], and also washed erythrocytes of sheep, ox and horse, laked by freezing and freed from stroma by adjusting the *p*H to 5·8 and keeping in the refrigerator. All these solutions behaved qualitatively in the same way.

At first the solutions were Seitz-filtered and the experiments carried out under strictly sterile conditions, but this precaution was later found to be unnecessary for experiments up to 2 hr. incubation time. No signs of bacterial contamination were observed and the rate of formation of the choleglobin absorption band was the same as in experiments under sterile conditions.

The haemoglobin concentration varied between 0·3 and 3 %, the majority of experiments being carried out with a haemoglobin solution of about 0·7 %. Crystalline ascorbic acid (B.D.H.) was used. Its concentration in the reaction mixture was usually 100 mg. per 100 ml. The solutions were buffered with phosphate; phosphate is not necessary for the reaction with ascorbic acid, choleglobin being formed without the addition of buffers, or with borate buffers. The

---

[1] The work was carried out under a grant from the National Health and Medical Research Council of Australia.

majority of experiments were carried out either at *p*H 7·2, which was obtained by adding 20 ml. of *M*/20 phosphate buffer *p*H 6·6 to 30 ml. of a 1·2 % haemoglobin solution, or at *p*H 8·5 obtained by adding 5 ml. of a solution of disodium hydrogen phosphate saturated at room temperature (20°) to 45 ml. of an 0·8 % haemoglobin solution. The final haemoglobin concentration was thus 0·72 %.

The reaction mixtures were incubated at 37° in flat culture flasks exposed to atmospheric oxygen without shaking in a layer of 4 mm. depth. A few experiments were carried out in Warburg vessels with shaking.

Spectroscopic observations

Choleglobin. Under these conditions the absorption band of reduced choleglobin at 628–630 mμ appears a few minutes after the reaction has been started by the addition of ascorbic acid. There was no noticeable formation of reduced haemoglobin and the bands of oxyhaemoglobin remained visible. The band in the orange can be due neither to methaemoglobin nor to methaemalbumin [Fairley, 1938; 1939], since addition of $Na_2S_2O_4$ does not destroy it, but increases its strength. Alkali shifts the band to 618–622 mμ, transforming choleglobin into denatured globin-cholehaemochromogen. If pyridine is added to the choleglobin solution until the precipitate of denatured globin-haemochromogens is redissolved and $Na_2S_2O_4$ added at once, there is also only the band of cholehaemochromogen at 618–620 mμ beside the bands of protohaemochromogen in the green. $Na_2S_2O_4$ must be added without delay in order to stop the formation of verdohaemochromogen from pyridine protohaemochromogen by coupled oxidation with the excess of ascorbic acid.

During the incubation the absorption band of reduced choleglobin gradually increases in intensity, but later, after about 30 min. of incubation, it is replaced by an absorption band at about 670 mμ. On reduction with $Na_2S_2O_4$ this band gives place to the band of reduced choleglobin at 630 mμ; it is therefore due either to ferric choleglobin or to oxycholeglobin. Carbon monoxide also causes the disappearance of the band at 670 mμ and the formation of a strong band at 628 mμ. The position of this band is very close to that of reduced choleglobin and may lead to the assumption that CO acts only as an inert gas allowing the excess ascorbic acid to reduce the oxidized choleglobin. It can, however, be proved that a CO-choleglobin is formed. Shaking with a mixture of 20 % O_2 and 80 % CO causes the band at 670 mμ to be replaced by that at 628 mμ, while shaking with air leaves the band at 670 mμ unaltered. CO also reacts with the alkaline solution of cholehaemochromogen shifting the absorption band from 618 mμ to 628 mμ. CO-choleglobin and CO-cholehaemochromogen thus have their absorption bands in the same position, just as do CO-haemoglobin and CO-protohaemochromogen. In the chole-series, however, the CO-compound has an absorption very similar to that of the reduced choleglobin, while in the proto-series the bands of the CO-compound differ widely from that of reduced haemoglobin.

These observations do not offer sufficient proof that the oxidized form of choleglobin is oxycholeglobin with O_2 reversibly bound to ferrous choleglobin. Ascorbic acid is still present in the solution and CO may not react directly with the oxidized compound, but by combining with ferrous choleglobin it may shift an equilibrium between ferric and ferrous forms, normally much in favour of the ferric form, in favour of the ferrous form, until the formation of CO-choleglobin is practically complete. Experiments carried out in order to decide whether the oxidized form of choleglobin is ferric or contains O_2 reversibly bound, will be described later.

Cholehaemochromogen. After about 45 min. incubation a green precipitate begins to form as a bottom layer and as a thin surface skin. We shall call this precipitate 'green pigment'; it has been previously described by Anderson & Hart [1934] and by Edlbacher & v. Segesser [1937]. After 80 min., more green pigment has been formed which, up to this stage, is almost free from protohaemochromogen. It is insoluble in water, soluble in dilute alkali, 1 % acetic acid or pyridine. These solutions are olive-green. On reduction with $Na_2S_2O_4$ they turn pure green, but seen against artificial light the colour is brown. The absorption spectrum of the reduced alkaline solution shows, beside weak protohaemochromogen bands, the band of ferrous cholehaemochromogen at 616–618 mμ, which is shifted by CO to 628 mμ. In pyridine the band lies at 619 mμ, while in dilute acetic acid or in neutral aqueous suspension the band is found at 628 mμ. Green pigment consists thus mainly of denatured globin choleparahaematin (ferric cholehaemochromogen). The absorption spectrum of the unreduced ferric compound varies with the conditions. In pyridine there is a broad band with its centre at about 612 mμ and a second band at 665 mμ. This second band is not that of verdohaemochromogen, since it is abolished by $Na_2S_2O_4$ reduction, while the similar absorption band of verdohaemochromogen is not altered by $Na_2S_2O_4$ in neutral pyridine solution. In 1 % Na_2CO_3 there is only an indistinct absorption band at about 595 mμ, while in *N*/15 NaOH a sharp band at 646·5 mμ appears. The solution in dilute acetic acid displays an absorption band at about 610 mμ.

Although the absorption spectrum of ferrous cholehaemochromogen in neutral suspension or in weakly acid solution does not differ from that of choleglobin, the two compounds differ totally in solubility. Cholehaemochromogen is insoluble at neutral reaction and is quantitatively precipitated from its alkaline or acid solutions by neutralization or by dialysis against distilled water. Choleglobin is soluble at neutral reaction and this solubility is not due to the protective colloid action of admixed haemoglobin. A haemoglobin solution of *p*H 7·2 or 8·5 does not dissolve green pigment; this can be quantitatively removed by filtration and no choleglobin band is observed in the filtrate. Choleglobin, however, can be redissolved in water after precipitation with ammonium sulphate, and can be absorbed on aluminium hydroxide and eluted from it by a phosphate buffer of *p*H 8·5.

Denaturation of choleglobin. It is of interest that there is no denaturation of oxyhaemoglobin under the conditions of our experiment, if ascorbic acid is absent. Two explanations of the denaturation leading to green pigment are possible. Either choleglobin is less stable than haemoglobin and undergoes spontaneous denaturation similar to that of free native globin, or the denaturation is due to an oxidation of the globin part of the haemoglobin molecule accompanying the oxidation of the prosthetic group. After the removal of ascorbic acid, choleglobin, though undergoing secondary changes to be described later, does not appear to be very readily denatured. It might be assumed, however, that the replacement of the four groups of haemoglobin by cholehaem groups does not occur simultaneously and that choleglobins with one or two bile pigment-haem groups are stable, while a choleglobin with all four haem groups replaced by cholehaem would undergo spontaneous denaturation. We have recently shown [Lemberg, Norrie & Legge, 1939] that catalase is probably a compound with three protohaematin and one bile pigment-haematin group per molecule. We were indeed, unable to obtain a choleglobin solution with more than 60 % cholehaematin and less than 40 % of protohaematin.

However this may be, the second explanation certainly also holds. Holden and his collaborators [Holden, 1936; 1937; Rawlinson, 1939] have repeatedly described oxidative denaturations of haemoglobin. Lemberg & Legge [1938] have shown that a part of the oxygen evolved from oxyhaemoglobin by acids is taken up through oxidation of the protein part of the molecule. While up to 80 min. incubation with ascorbic acid there is little denaturation of haemoglobin, after 2 hr. the precipitate contains a considerable proportion of ferric denatured globin-protohaemochromogen (cathaemoglobin), and the longer the reaction proceeds, the more haemoglobin is denatured. There appear to be also further changes in the nature of the protein in the precipitate; its solubility in alkali and pyridine decreases. Finally, as will be described in the fifth paper of this series, the prosthetic groups, both protohaem and cholehaem, are bound to the protein of the precipitate much more firmly than in the choleglobin solution or in the haemochromogens produced from it by alkali denaturation.

These observations enhance the steadily increasing amount of evidence that the products obtained by 'denaturing' one and the same protein by different methods may differ considerably (cf. the discussion of this problem in the *Cold Spring Harbor Symposia on Quantitative Biology* [1938]).

Products of the later stages of the reaction and of further oxidation of cholehaemochromogen

After incubation for several hours there is a great deal of precipitate, now more brown than green. The supernatant solution contains oxidized choleglobin, but the band at 670 mμ no longer disappears completely on reduction with $Na_2S_2O_4$ or with CO. On addition of pyridine and $Na_2S_2O_4$ an absorption band at 665 mμ, in addition to the cholehaemochromogen band at 618 mμ, appears even if $Na_2S_2O_4$ is added at once to the pyridine solution. The compound which causes the band at 665 mμ may be verdohaemochromogen, and verdoglobin[1] may be present in the supernatant solution before addition of pyridine. Addition of alkali to the reduced solution decreases the strength of the band in the red, and reduced verdoglobin behaves in a similar way, being transformed into a yellow compound. The strength of the cholehaemochromogen band at 618 mμ appears, however, to be increased by the addition of alkali; verdoglobin is not transformed into cholehaemochromogen by alkali, and if the increase of the band at 618 mμ accompanying the decrease of the band in the red is due to a transformation of the compound causing this band into cholehaemochromogen, the compound cannot be verdoglobin.

A substance with a similar absorption band in the red is produced if a pyridine solution of 'green pigment', which contains practically no protohaemochromogen, is left standing in the presence of air and ascorbic acid. Brown solutions are obtained which on reduction with $Na_2S_2O_4$ no longer display the absorption band of cholehaemochromogen, but a band at 657 mμ and a weaker band at 534 mμ. This compound is not formed if the ascorbic acid is washed away from the 'green pigment' before dissolving it in pyridine, or if the solution is left standing *in vacuo*. There can be no doubt, therefore, that the new compound is an oxidation product of cholehaemochromogen, formed from it by coupled oxidation with ascorbic acid in pyridine. In its absorption spectrum it resembles verdohaemochromogen and also in its behaviour towards $Na_2S_2O_4$ in alkaline solution. Nevertheless its identity with verdohaemochromogen is doubtful. The latter is green and gives rise to yellow compounds only by the action of $Na_2S_2O_4$ in alkali, while the colour of the oxidation product from cholehaemochromogen is brown in the ferric state as well as when reduced in neutral pyridine solution.

---

[1] Cf. Lemberg & Wyndham [1937, p. 352]. We prefer now to call the compound of verdohaem with native globin verdoglobin in spite of the fact that it does not reversibly unite with oxygen.

Absorption curves

The absorption curves of choleglobin and its derivatives were investigated with a Visible Absorption Spectrophotometer of Adam Hilger, consisting of the Barfit Hilger Constant Deviation Wavelength Spectrometer with prism n_D 1·62 and the Barfit Hilger Nutting Photometer on the Standard Accessory Bar.

Fig. 1 shows the absorption curves in the region from 740 to 600 mμ of the oxygenated choleglobin solution (full-drawn line), that of the same solution after reduction with $Na_2S_2O_4$ (dotted line) and again after saturation with coal gas (interrupted line). The absorption curves in the region of 600–500 mμ were mainly those of the accompanying haemoglobin compounds, the maximal density of the CO-haemoglobin at 570 mμ being 1·63 times as strong as that of the CO-choleglobin at 630 mμ. The absorption band of the oxygenated solution at 670 mμ is far less marked than when viewed through a pocket spectroscope. Reduced choleglobin and CO-choleglobin both show a strong absorption band at 630 mμ, that of the CO-compound being somewhat sharper. Their absorptions in the wave-length region of 700–655 mμ are markedly less than that of oxygenated choleglobin.

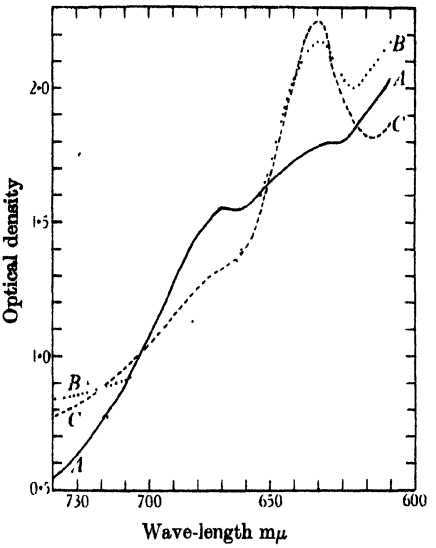


Fig. 1.

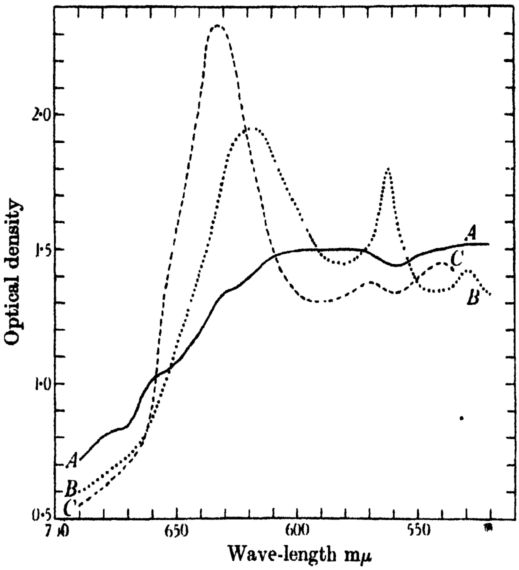


Fig. 2.

Fig. 1. Choleglobin. Curve *A* (full line), oxygenated. Curve *B* (dotted line), reduced. Curve *C* (interrupted line), CO.

Fig. 2. Cholehaemochromogen. Curve *A* (full line), green pigment in 1% Na_2CO_3. Curve *B* (dotted line), reduced with $Na_2S_2O_4$. Curve *C* (interrupted line), reduced with $Na_2S_2O_4$ in coal gas.

The absorption curves of some cholehaemochromogen derivatives are given in Fig. 2. (The fully drawn line represents the absorption curve of green pigment dissolved in 1 % Na_2CO_3, the dotted line that of the ferrous cholehaemochromogen obtained by reduction with $Na_2S_2O_4$.) CO without reducer has no effect on the absorption curve of ferric cholehaemochromogen, but in the presence of $Na_2S_2O_4$ the band of the ferrous CO-cholehaemochromogen at 628 mμ appears which is stronger and sharper than that of the CO-free compound. The opposite is true with regard to the bands of protohaemochromogen at 559 mμ and of CO-protohaemochromogen at 570 mμ.

From the appearance of these maxima in the absorption curves of 'green pigment' derivatives it is evident that green pigment contains some protohaemochromogen. The ratio $\epsilon_{630} : \epsilon_{570}$ of its CO-haemochromogen was 1·70. We have obtained other preparations with a higher ratio, the highest being 2·08. The last-mentioned preparation gave only a just noticeable hump at 560 mμ due to ferrous protohaemochromogen. If we assume as a first approximation: ϵ_{570} of cholehaemochromogen $= \epsilon_{570}$ of CO-cholehaemochromogen $= \epsilon_{557}$ of cholehaemochromogen $= \epsilon_{557}$ of CO-cholehaemochromogen $= x$, and ϵ_{570} of CO-protohaemochromogen $= y$, we have two equations with two unknowns:

$$\epsilon_{570}^{CO} = x + y, \quad \epsilon_{557} = x + 2\cdot14y,$$

the factor 2·14 being the measured ratio ϵ_{557} of protohaemochromogen to ϵ_{570} of CO-protohaemochromogen. If we substitute the values of ϵ_{570}^{CO} and ϵ_{557} from the curves in Fig. 2, we obtain $x = 0\cdot99$ and $\epsilon_{630} : \epsilon_{570}$ of CO-cholehaemochromogen $= 2\cdot33 : 0\cdot99 = 2\cdot35$. From this figure and the highest figure actually observed, we may safely conclude that 2·22 for this ratio is a good approximation; this figure will be used in Part 3 for establishing the absolute absorption values of choleglobin and cholehaemochromogen.

Does choleglobin combine reversibly with oxygen?

The question whether or not choleglobin unites reversibly with oxygen has an important bearing on the problem of the 'labile iron' in blood which we shall discuss in Part 4. We have therefore studied it in some detail.

A solution of choleglobin, obtained by 90 min. incubation of oxyhaemoglobin with 100 mg. per 100 ml. ascorbic acid at *p*H 8·5 under the conditions described above, was left at room temperature for 16 hr. in order to complete the oxidation of ascorbic acid, and then filtered. This solution failed to show any oxygen uptake in a Warburg respirometer during 10 min., but still contained small amounts of indophenol-reducing substance in the trichloroacetic acid filtrate (less than 3 mg. ascorbic acid per 100 ml.). Fig. 3 shows the effect of coal gas with acid but without added $Na_2S_2O_4$ on the absorption curve of the choleglobin (oxygenated solution fully drawn line). CO without reducer (interrupted line) still causes the appearance of a distinct absorption maximum at 630 mμ and a decrease of absorption at 670 mμ, but the effect of CO plus reducer is more marked. Similar results were obtained with a solution of choleglobin dialysed for 16 hr. against distilled water. On exhaustion of a solution of CO-choleglobin in a Thunberg tube, followed by reoxygenation, the band at 630 mμ disappeared and was again replaced by the band at 670 mμ. These observations lend some support

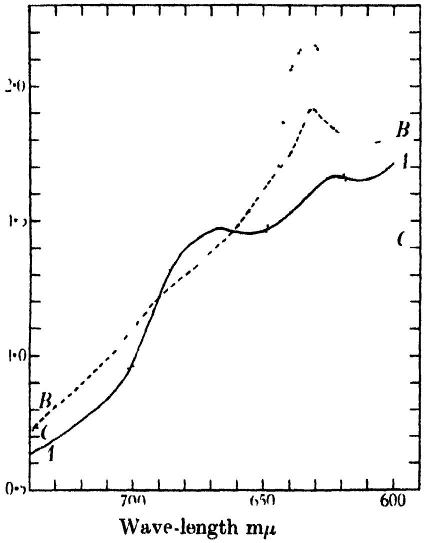


Fig. 3. Reaction of choleglobin with carbon monoxide. Curve *A* (full line), oxygenated. Curve *B* (interrupted lines), in coal gas. Curve *C* (dotted line), in coal gas with $Na_2S_2O_4$.

to the assumption that the oxygenated form of choleglobin reacts with CO and is, therefore, ferrous oxycholeglobin, with oxygen bound reversibly as in oxyhaemoglobin. The presence of small amounts of reducing substances in the solution does not, however, allow a definite conclusion.

We have attempted to remove the last traces of reducing substances by prolonged dialysis or by precipitation with ammonium sulphate followed by careful washing of the precipitate with saturated ammonium sulphate solution. Solutions were thus obtained which no longer reacted with CO without $Na_2S_2O_4$ (Fig. 4). The oxygenated solution, however, failed now to show the typical absorption band at 670 mμ of the original oxygenated choleglobin. During the dialysis some methaemoglobin was formed from oxyhaemoglobin, and if oxycholeglobin exists, it may be more easily transformed into a metacholeglobin than oxyhaemoglobin into methaemoglobin and thus lose its property of combining with CO without $Na_2S_2O_4$. This view is supported by the observation that ferricyanide destroyed the band at 670 mμ and decreased the absorption in this wave-length region (Fig. 5), but we failed to restore the band at 670 mμ by reduction of the ferricyanide-treated solution with an amount of $Na_2S_2O_4$ just sufficient to reduce the accompanying methaemoglobin to reduced haemoglobin and subsequent oxygenation; oxyhaemoglobin could be thus reconstituted.

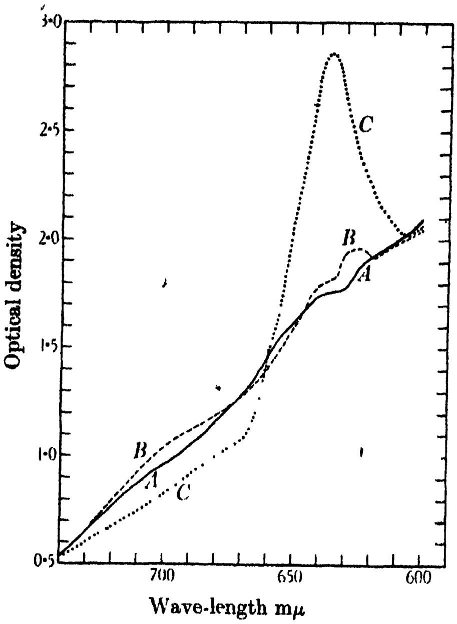


Fig. 4.

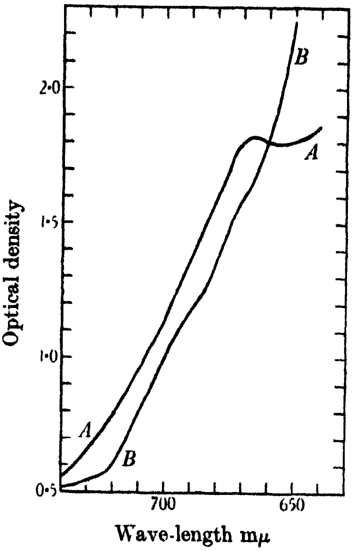


Fig. 5.

Fig. 4. Dialysed choleglobin and carbon monoxide. Curve *A* (full line), oxygenated. Curve *B* (interrupted line), in coal gas. Curve *C* (dotted line), in coal gas with $Na_2S_2O_4$.

Fig. 5. Effect of ferricyanide on oxygenated choleglobin. Curve *A*, oxygenated choleglobin. Curve *B*, the same after addition of ferricyanide.

Evacuation of a fresh choleglobin solution also caused the absorption band at 670 mμ to disappear and produced the band of reduced choleglobin, but the reaction proceeded more slowly than the transformation of oxyhaemoglobin into reduced haemoglobin. Reoxygenation yields again the band at 670 mμ. In long-dialysed choleglobin solutions evacuation had no effect.

Another attempt was made starting from ferric choleglobin cyanide, the preparation of which is described below. Cyanide was removed by precipitation with ammonium sulphate from a solution reduced with $Na_2S_2O_4$ and by washing the precipitate with a saturated ammonium sulphate solution containing some phosphate buffer *p*H 7·6 and some $Na_2S_2O_4$. The precipitate was dissolved in water and the resulting solution reoxygenated. In this way the accompanying cyanmethaemoglobin was transformed into oxyhaemoglobin. There was an absorption band at about 680 mμ and CO failed to produce the band of CO-choleglobin without addition of $Na_2S_2O_4$. The band at 680 mμ differed, however, from that of the oxygenated choleglobin solution produced by ascorbic acid by not being removed by $Na_2S_2O_4$; it is, therefore, due to a ferrous haematin compound different from choleglobin, and not to oxygenated choleglobin. Again this throws no light on the nature of the compound responsible for the band at 670 mμ in oxygenated choleglobin solutions. Since we failed in our attempts to remove the last traces of reducing substances from choleglobin solutions without altering the oxygenated compound, we cannot decide whether this is metacholeglobin or oxycholeglobin; if oxycholeglobin exists, it must be more labile than oxyhaemoglobin.

Action of hydrogen peroxide on haemoglobin in the presence of cyanide

By the action of hydrogen peroxide on oxyhaemoglobin solutions containing potassium cyanide Barkan & Schales [1938] have obtained a green solution showing the following absorption spectrum after reduction with $Na_2S_2O_4$: I, 617–620, II, 564–568, III, 536–540 mμ. In repeating these experiments we found concentrations of 2% cyanide and 0·05–0·08% hydrogen peroxide optimal for the transformation into the green compound. The authors recognized that the absorption spectrum is due to the presence of two compounds, one causing the absorption band in the orange and the other the two bands in the green. The first substance they call 'pseudohaemoglobin' assuming that it consists of a bile pigment-iron complex bound to native globin. About the nature of the second substance they offer no definite suggestions, but apparently they believe that it is another product of oxidation of haemoglobin, no longer containing protohaem.

We will discuss this compound first, since the understanding of its nature will help us to understand that of the compound with the band at 617–620 mμ. The two bands in the green are those of ferrous denatured globin-cyan-protohaemochromogen. The bands can be produced without application of hydrogen peroxide by keeping an oxyhaemoglobin solution containing 2% cyanide 24 hr. at room temperature. Before reduction only one band at 542 mμ is seen which is that of cyancathaemoglobin (denatured globin-ferric protohaemochromogen cyanide). The denaturation of the globin is caused by the alkalinity of the cyanide. In the presence of hydrogen peroxide there is a more rapid oxidative denaturation, complete in 20 min. as shown by the change of the absorption spectrum of oxyhaemoglobin into that of cyancathaemoglobin. On careful neutralization or on dialysis against a neutral buffer complete precipitation occurs. Both the 'pseudohaemoglobin' and the compound with the bands in the green contain, therefore, denatured globin and not native globin.

Barkan & Schales have overlooked the identity of their second compound with ferrous cyanprotohaemochromogen probably because they observed a few spectroscopic properties, which hitherto have not been described for ferrous cyanprotohaemochromogen. In the weakly cyanide-alkaline solution and faster still in a neutral suspension or in a weakly acid solution the bands at 566 and

536 mμ of ferrous cyanprotohaemochromogen disappear and are replaced by a single rather diffuse band at 558 mμ, while in a more strongly alkaline solution they remain unaltered. NaOH added to the solution with the diffuse band at 558 mμ restores the original double-banded spectrum. As far as we know, the compound with the single band has so far not been described and its nature is unknown.[1]

Under conditions in which the single-banded compound arises from ferrous cyanprotohaemochromogen, CO transforms the latter at once into CO-protohaemochromogen (absorption bands at 570·5 and 540 mμ), while it does not react with it in strongly alkaline solutions. In a weakly cyanide-alkaline solution the position of the bands indicates an equilibrium between CO- and CN-compounds, which depends on the cyanide concentration and on the *p*H of the solution.

Under all these conditions the absorption bands in the green produced by the action of hydrogen peroxide behave exactly in the same manner as the bands of cyanprotohaemochromogen.

These observations in the protohaematin series allow us to interpret the nature of 'pseudohaemoglobin' and to explain the changes of its absorption studied by Barkan & Schales in a simple and more correct way. Dialysis, which removes the cyanide, transforms the compound into a substance with all the properties of cholehaemochromogen. 'Pseudohaemoglobin' differs from cholehaemochromogen in not reacting with CO in strongly alkaline solution reduced by $Na_2S_2O_4$; while coal gas shifts the band of cholehaemochromogen from 618 to 628 mμ, the band of 'pseudohaemoglobin' at 618 mμ remains unaltered. The compound is, therefore, a cyanide-compound, a conclusion also reached by Barkan. Evidently the substance is ferrous denatured globin cyancholehaemochromogen. Its behaviour towards CO is very similar to that of the corresponding protohaematin compound which accompanies it; it reacts with it only in acid or in weakly alkaline solution, in the latter somewhat less completely than the protohaematin compound.

Barkan & Schales believe that the dialysis of 'pseudohaemoglobin' causes an essential alteration changing 'pseudohaemoglobin' into 'pseudohaemochromogen'. If these names were taken at their face value, this would mean that the dialysis causes a denaturation of protein, which cannot be correct, the protein in 'pseudohaemoglobin' being already denatured. If we understand the authors correctly, they mean a slight alteration of the prosthetic group. Their evidence is based on their observations that the fresh cyanide-alkaline solution shows a shift of the absorption band with CO towards the infra-red while a dialysed solution to which the same amount of cyanide has been added, fails to show this shift. This is further complicated by another observation that if the order is reversed and the dialysed compound first transformed into the CO compound (band at 628 mμ), cyanide added now fails to shift the band back to 618 mμ. We cannot confirm these rather confusing observations. Provided that *p*H and cyanide concentration were actually restored, the dialysed solution to which cyanide had been added behaved exactly as the undialysed solution. Barkan's observation may be explained as follows: the fresh solution still contains some excess hydrogen peroxide and its reaction with $Na_2S_2O_4$ tends to acidify the solution, shifting the equilibrium between CO- and CN-compounds in favour of the former. We have failed to observe the final equilibrium to vary with the sequence in which the reagents were added, provided that sufficient time was allowed for the equilibrium to establish itself.

---

[1] Dicyanprotohaemochromogen, obtained by dissolving haemin in 10% KCN and adding $Na_2S_2O_4$ (absorption bands I, 572·4, II, 539·8 mμ, intensities II, I), can also be transformed into a similar single-banded compound with an absorption band at 555 mμ, if much $Na_2S_2O_4$ is added to a very dilute solution in water; the effect is due to the acidifying action of $Na_2S_2O_4$, alkali restoring again the two-banded spectrum.

The globin denaturation during the action of hydrogen peroxide can be avoided by neutralizing the cyanide. When 0·05 % hydrogen peroxide acts on haemoglobin in the presence of 1 % KCN neutralized to litmus by KH_2PO_4, a compound with an absorption band in the red (at about 680 mμ) is formed which is soluble at *p*H 7·2. The band lies further towards the infra-red and is more diffuse than that of oxygenated choleglobin. Reduction with $Na_2S_2O_4$ produces reduced choleglobin. The compound does not react with CO without reducer, but yields CO-choleglobin after addition of $Na_2S_2O_4$. It is evidently cyanmeta-choleglobin (ferric choleglobin cyanide); a second absorption band in the green is that of cyanmethaemoglobin.

Attempts to isolate choleglobin from haemoglobin

Two different methods were tried, fractional precipitation with ammonium sulphate and differential adsorption on alumina. By the former no separation whatever was obtained. The ratio $\epsilon_{628} : \epsilon_{570}$ of the CO-haemochromogens was the same in the original solution, the filtrate and the solution of the precipitate.

When a solution with the ratio $\epsilon_{628} : \epsilon_{570}$ of the CO-haemochromogens = 0·37 was adsorbed on Willstätter's alumina C, the filtrate had a ratio of 0·36, the eluate from the alumina with 0·1 % ammonia a ratio of 0·405. The latter was brought to *p*H 6 by addition of acetate buffer *p*H 4·6 and again partially adsorbed on alumina. The ratio in the filtrate was this time 0·30 and in the eluate from the precipitate 0·50, but the eluate was slightly cloudy. Similar results were obtained with Brockmann's aluminium hydroxide (Merck) and with Fasertonerde according to Wislicenus (Merck). Choleglobin appears to be adsorbed preferentially, but the difference is too slight to encourage attempts at complete separation.

SUMMARY

The preparation of choleglobin and cholehaemochromogen by the coupled oxidation of haemoglobin and ascorbic acid and the absorption spectra of these compounds are described. The oxidation of the haem part of the molecule, causing the formation of choleglobin, is soon followed by an oxidative denaturation of the globin part, leading to cholehaemochromogen.

Choleglobin and cholehaemochromogen reversibly combine with CO. Whether or not choleglobin combines reversibly with O_2, could not be established.

Cholehaematin is distinct from verdohaematin, although verdohaemochromogen is formed in the same type of reaction from pyridine-protohaemochromogen as is choleglobin from haemoglobin.

The 'pseudohaemoglobin' of Barkan & Schales, prepared by action of hydrogen peroxide and potassium cyanide on oxyhaemoglobin followed by reduction with sodium hydrosulphite, is ferrous denatured globin-cyan-cholehaemochromogen. With neutralized cyanide cyanmetacholeglobin is obtained. The solutions, however, contain by-products.

No effective way of separating choleglobin from haemoglobin was found.

Further oxidation of choleglobin and of cholehaemochromogen leads to compounds which in their absorption spectra resemble verdohaematin compounds, but which differ from them in some properties. Such products are also formed in the course of prolonged coupled oxidation of haemoglobin and ascorbic acid.

The spectrophotometer used in this research was kindly provided by the National Health and Medical Research Council of Australia, to which two of us are also indebted for grants. One of us is working with support from the T. E. Rofe bequest.

REFERENCES

Anderson & Hart (1934). *J. Path. Bact.* **39**, 465.
Barkan & Schales (1937). *Hoppe-Seyl. Z.* **248**, 96.
—— —— (1938). *Hoppe-Seyl. Z.* **253**, 83.
Edlbacher & v. Segesser (1937). *Naturwissenschaften*, **25**, 461, 557, 667.
Fairley (1938). *Nature, Lond.*, **142**, 1156.
—— (1939). *Proc. roy. Soc. Med.* **32**, 1278.
Heidelberger (1922). *J. biol. Chem.* **53**, 31.
Holden (1936). *Aust. J. exp. Biol. med. Sci.* **14**, 291.
—— (1937). *Aust. J. exp. Biol. med. Sci.* **15**, 43.
Lemberg & Wyndham (1937). *J. roy. Soc. N.S.W.* **70**, 343.
—— Cortis-Jones & Norrie (1938). *Biochem. J.* **32**, 149.
—— & Legge (1938). *J. roy. Soc. N.S.W.* **72**, 62.
—— —— & Lockwood (1938). *Nature, Lond.*, **142**, 148.
—— —— —— (1939). *Biochem. J.* **33**, 754.
—— Norrie & Legge (1939). *Nature, Lond.*, **144**, 551.
Rawlinson (1939). *Aust. J. exp. Biol. med. Sci.* **17**, 53.
Symposia on Quantitative Biology, Biological Laboratory, Cold Spr. Harb. Vol. **6** (1938).

34. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN

3. QUANTITATIVE STUDIES ON CHOLEGLOBIN FORMATION. ESTIMATION OF HAEMOGLOBIN AND ASCORBIC ACID OXIDATIONS[1]

By R. LEMBERG, J. W. LEGGE and W. H. LOCKWOOD

From the Institute of Medical Research, Royal North Shore Hospital, Sydney

(*Received 3 March 1941*)

In this paper we describe a spectrophotometric method allowing us to measure quantitatively the destruction of haemoglobin and the formation of choleglobin and cholehaemochromogen during the coupled oxidation of haemoglobin with ascorbic acid and with other reducing substances. The influence of various factors, the nature of the reactants, *p*H, temperature and oxygen pressure, on the velocity of the reaction is studied. The oxidation of ascorbic acid during the reactions is measured in the usual way by indicator titration, and the ratio of molecules of ascorbic acid oxidized to molecules of choleglobin formed is thus found. The spectrophotometric determination method enables us, also, quantitatively to correlate the formation of choleglobin with the increase of 'labile iron' and with the yield of bile pigments to be obtained after acid splitting, thus giving us a much more complete insight into the reaction; the results of these studies are described in Parts 4 and 5.

EXPERIMENTAL

Spectrophotometric determination of choleglobin formation

Technique. A large number of difficulties had to be overcome before a reliable technique could be developed. The solutions derived from the incubation of oxyhaemoglobin and ascorbic acid contain a mixture of oxyhaemoglobin, a little reduced haemoglobin, in some instances methaemoglobin, and reduced and oxygenated choleglobin; in the later stages of the reaction the precipitate of green pigment appears and even in the earlier stages a slight cloudiness occasionally develops. It is evident, therefore, that spectrophotometric study of the absorption of the solutions as such is useless. Means had to be devised to transform the various derivatives of protohaematin and of cholehaematin into one derivative of each and to ensure an optically clear solution. The transformation into the CO-haemochromogens by CO, NaOH and $Na_2S_2O_4$ fulfilled these demands. The CO-free haemochromogens are less suitable, being less stable. Even in the presence of CO we found it necessary to work under strictly controlled conditions in order to get reproducible results. Since $Na_2S_2O_4$ and O_2 yield peroxide which produces choleglobin, it is essential to add the reducer only after the solution has been saturated with coal gas and even then to avoid unnecessary exposure to air. We found it also necessary to add the alkali only after the solution had been saturated with coal gas. A CO-haemoglobin solution, which had an extinction coefficient at 570 mμ of 1·0, gave as CO-haemochromogen

---

[1] This work was carried out under a grant from the National Health and Medical Research Council of Australia.

produced by addition of alkali and $Na_2S_2O_4$ after saturation with coal gas the extinction coefficients at 570 mμ: 1·00; 1·01; 0·98; 0·99. When alkali was added before the saturation with coal gas, the values were smaller and the variation greater: 0·81; 0·91; 0·80; 0·90; 0·91.

The following method was employed. 5 ml. of the solution to be investigated were at once saturated with coal gas for 3 min. The current of gas was as rapid as the frothing of the solution allowed. Only in a few instances did we find it necessary to add a trace of octyl alcohol, which tends to cause some denaturation. 50 mg. of $Na_2S_2O_4$ were then added, the tube once inverted and during further passage of coal gas 0·5 ml. of 20 % NaOH was introduced. A vigorous current of coal gas was maintained for 1 min., after which the solution was transferred to the spectrophotometer screw cups. The extinction coefficients at 630 and 570 mμ were then measured without delay, although we found the absorption of the solutions in the cups to change only very slowly. The dilution to the concentration suited for spectrophotometric measurement must be carried out before the addition of $Na_2S_2O_4$ and alkali; the dilution by the alkali has, of course, to be considered. The instrument used was the Hilger Visible Spectrophotometer described in the preceding paper.

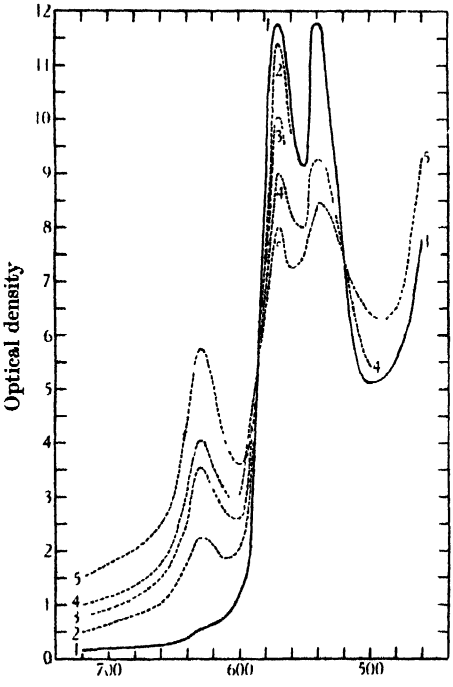


Fig. 1. Formation of choleglobin by coupled oxidation of haemoglobin and ascorbic acid. CO-haemochromogens. Curve 1: from haemoglobin. Curves 2, 3, 4 and 5: after 15, 30, 60 and 120 min. coupled oxidation at 37° respectively.

Changes of the absorption curves of the CO-haemochromogens during the incubation of oxyhaemoglobin and ascorbic acid

Fig. 1 shows the changes of the absorption curves of the CO-haemochromogens during incubation of a 1·2 % oxyhaemoglobin solution with 100 mg. per 100 ml. of ascorbic acid at 37° and *p*H 8·5 in a culture flask without shaking (cf. Part 2).

The continuous line represents the curve of CO-protohaemochromogen, the interrupted lines those of the CO-haemochromogens obtained after varying times of incubation with ascorbic acid. The absorption of the CO-cholehaemochromogen at 630 mμ rises, while that of the CO-protohaemochromogen at 570 and 540 mμ decreases, the decrease of absorption at 540 mμ being somewhat less than at 570 mμ. Two isosbestic points are observed at 585 and 520 mμ. Owing to the steep course of the CO-protohaemochromogen curve at these wavelengths the isosbestic point cannot be determined very accurately, but the curves display no evidence of the formation of more than one oxidation product during the duration of this experiment (120 min.).

Quantitative estimation of choleglobin

In order to determine the choleglobin concentration in a mixture with haemoglobin from the absorption of the CO-haemochromogens at 630 mμ it is necessary to know the specific extinction coefficients of CO-cholehaemochromogen at this wave-length. Pure CO-cholehaemochromogen has not yet been obtained; we can, however, calculate the specific extinction of CO-cholehaemochromogen from the measured ratio between the decrease of extinction at 570 mμ and the increase at 630 mμ if during the incubation only those compounds are formed which yield CO-cholehaemochromogen or CO-protohaemochromogen with CO, NaOH and $Na_2S_2O_4$. The data reported in the previous section indicate that this is the case, at least in the initial stages of the incubation. It is also necessary to know the ratios of the extinction coefficients at 630 and 570 mμ ($\epsilon_{630} : \epsilon_{570}$) of both CO-cholehaemochromogen and CO-protohaemochromogen. The latter ratio can be determined; the absorption of CO-protohaemochromogen at 630 mμ is small and the ratio $\epsilon_{630} : \epsilon_{570}$ for CO-protohaemochromogen (0·04 as average of a large number of determinations) has only the character of a correction factor. The ratio for CO-cholehaemochromogen cannot be determined correctly, but we arrived at the conclusion that it cannot be far from 2·22 (cf. p. 333, Part 2). If we use these values, the ratio ϵ_{630} of CO-cholehaemochromogen to ϵ_{570} of CO-protohaemochromogen, R, is obtained by the equation:

$$R = \frac{Q - 0{\cdot}04}{1 - Q/2{\cdot}22},$$

where Q is the observed ratio $\epsilon_{630} : \epsilon_{570}$. R ought to remain constant during the course of the reaction, if our assumption that cholehaematin derivatives are the only reaction products is correct. Table 1 shows that R is approximately 1·0 under widely varying conditions of the reaction. R does not remain absolutely constant during the reaction, but sufficiently so to indicate that at least during the early stages of the reaction choleglobin is practically the only product. Later, lower values of R are found, indicating some formation of by-products.

Table 1. *Values obtained for ratio (R) of ϵ_{630} of CO-cholehaemochromogen to ϵ_{570} of CO-protohaemochromogen from measurements of absorption decrease at 570 mμ and increase at 630 mμ*

*p*H 7·2, 37°		*p*H 8·5, 37°		*p*H 8·5, 20°	
Min. of incubation	*R*	Min. of incubation	*R*	Hr. of incubation	*R*
30	0·95	15	1·03	19½	0·96
40	0·91	30	1·08	31	0·84
80	0·83	40	0·96		
120	0·84	80	0·92		
		90	0·94		
		120	0·81		

For our calculations we assume $R=1$, i.e. equal maximal extinctions of CO-cholehaemochromogen and CO-protohaemochromogen. The specific extinction coefficient (extinction of a 0·1 % solution in 1 cm. layer) of CO-protohaemochromogen was found to be 0·965 at 570 mμ, and the same value is assumed for the specific extinction coefficient of CO-cholehaemochromogen at 630 mμ.

For the specific extinction coefficient at 630 mμ of the CO-cholehaemochromogen prepared from 'green pigment' we found the value 1·03 when the concentration was measured by the determination of the total iron in green pigment. A lower value (0·76) was obtained when the concentration was measured by weighing the protein denatured by formalin. Green pigment appears thus to contain some protein without strong absorption at 630 mμ and without iron. The correction for the protohaematin content in green pigment was based on the same data as the calculation of R from the ratio $\epsilon_{630} : \epsilon_{570}$, but the protohaematin content of green pigment is small and the correction therefore of much less importance. The agreement of the values obtained from green pigment with those obtained from choleglobin-haemoglobin solutions can, therefore, be taken as independent confirmation that the values arrived at for R and for the specific extinction coefficient at 630 mμ of CO-cholehaemochromogen are approximately correct.

We can now determine the content of cholehaematin and protohaematin derivatives in a solution. If ϵ°_{570} is the extinction coefficient at 570 mμ, of the haemoglobin solution before incubation with ascorbic acid, measured as CO-haemochromogen, and ϵ_{630} and ϵ_{570} the extinction coefficients at 630 mμ and 570 mμ after incubation, we have: choleglobin formed, in % of original haemoglobin $= \frac{102\,(\epsilon_{630} - 0{\cdot}04\,\epsilon_{570})}{\epsilon^{\circ}_{570}}$; and, unaltered haemoglobin $= \frac{102\,(\epsilon_{570} - 0{\cdot}45\,\epsilon_{630})}{\epsilon^{\circ}_{570}}$. If ϵ°_{570} is unknown, the choleglobin percentage of a mixture of choleglobin and haemoglobin is given by the equation: % choleglobin $= \frac{100\,(\epsilon_{630}/\epsilon_{570} - 0{\cdot}04)}{0{\cdot}96 + 0{\cdot}55\,\epsilon_{630}/\epsilon_{570}}$.

Estimation of protohaematin destruction

We have established the validity of the spectrophotometric method by estimating the destruction of protohaematin during the incubation of haemoglobin with ascorbic acid in a second way. By treatment of a haemoglobin solution containing ascorbic acid with acetic acid and ether, the protohaematin can be transferred almost quantitatively into the ether and can be determined spectrophotometrically as pyridine-protohaemochromogen in the ammoniacal extract. If this method is applied to choleglobin solutions, the ether contains beside protohaematin only biliverdin which is removed by extraction with 5 % HCl, and a small amount of biliviolin which does not disturb the haematin estimation.

For the determination the following technique was applied: 10 ml. of glacial acetic acid were added to 5 ml. of the choleglobin solution and the pigments were brought into 15 ml. of ether in the manner used for the determination of the bile pigments and described in detail in Part 5. The ether layer was repeatedly extracted with 5 % HCl, washed with water and finally the haematin was extracted with a few ml. of $N/10$ ammonia. Pyridine was added to a concentration of 20 % and after reduction with $Na_2S_2O_4$ the absorption at $\lambda = 557$ mμ (first absorption band of pyridine haemochromogen) was measured spectrophotometrically. The molar extinction coefficient of pyridine-protohaemochromogen at 557 mμ under these conditions was found to be 31,000.

We have made an interesting observation on the effect of ascorbic acid on the isolation of haematin from solutions of oxyhaemoglobin. 5 ml. of a 0·72 % solution of haemoglobin ought to yield 1·405 mg. haemin. If 5 mg. of ascorbic acid were added to the haemoglobin solution immediately before the acidification with glacial acetic acid, 1·35 mg. of haemin = 96 % were recovered from the ether. Without the addition of ascorbic acid 1·29 mg. = 92 % were recovered, when the ether was added immediately after the addition of the glacial acetic acid, when, however, the acetic acid solution was kept for 15 min. before the addition of ether and the separation of the layers, the aqueous layer contained a great deal of haematin firmly bound to protein and no longer extractable by ether, and the yield of haemin isolated from the ether was only 0·51 mg. or 36 % of the total haemoglobin-haemin. If acid haematin solution is kept in the presence of ascorbic acid, however, the yield of haematin does not diminish. Evidently ascorbic acid protects the globin in the acid solution from an alteration, probably of oxidative nature, which causes the protein-haematin linkage to become stable to acid.

Table 2. *Comparison of the spectrophotometric determination of choleglobin formation with the direct measurement of haemin destruction*

Conditions	mg. haemin from 5 ml haemoglobin	% haematin destruction	% choleglobin according to spectrophotometric method
Initial haemoglobin	1·35	0	0
*p*H 7·2, 15 min.	1·215	10·0	6·9
30 „	1·19	11·9	14·1
*p*H 8·5, 15 min.	1·08	20·0	17·9
30 „	0·905	33·0	33·3

Table 2 shows that the percentage destruction of protohaematin estimated by this method tallies with the percentage formation of choleglobin as determined by the spectrophotometric method. The rather large deviations are due to the fact that the estimation of haematin destruction is less exact than the spectrophotometric estimation of choleglobin formation.

Measurement of the velocity of choleglobin formation under various conditions

Table 3 gives the results of measuring the reaction velocity of the choleglobin formation with this method under various conditions of temperature and *p*H. Each of the values given is the average of at least three parallel experiments. The haemoglobin concentration was 0·72 %, that of ascorbic acid 100 mg. per 100 ml. and the incubation was carried out in flat culture flasks as previously described. As is to be expected from the nature of the experiment, variations are fairly large, but they do not affect the conclusions drawn from the experiments in any way. Table 4 shows the variation of a number of experiments carried out under the same conditions.

In the experiments in which a distinct precipitate of green pigment was found, a solution of the precipitate and the filtrate were analysed separately. The results of experiments of longer than 2 hr. can no longer be considered as reliable, since other compounds were formed in addition to choleglobin and cholehaemochromogen (cf. Part 2 and above). There is also occasionally bacterial contamination.

Table 3. *Choleglobin and cholehaemochromogen formation by coupled oxidation of haemoglobin with ascorbic acid*

		In % of initial haemoglobin					
Conditions	Duration of incubation	Choleglobin	Haemoglobin	Cholehaemochromogen	Protohaemochromogen	Total cholehaem	Sum
*p*H 7·2, 37°	15 min.	6·9	92·7	0	0	6·9	99·6
	30 ,,	14·1	83·1	0	0	14·1	97·2
	80 ,,	21·6	55·1	13·0	0·8	34·6	90·5
	120 ,,	17·2	46·2	21·5	2·7	38·7	87·6
	7 hr.	22·4	28·2	34·3	3·7	56·7	88·6
	16 ,,	11·5	13·6	—	—	—	—
*p*H 8·5, 37°	15 min.	17·9	83·2	0	0	17·9	101·1
	30 ,,	33·3	69·7	Trace included in former		33·3	103·0
	80 ,,	27·9	49·7	17·6	1·5	45·5	97·6
	120 ,,	36·5	32·9	20·3	2·2	56·8	91·9
	7 hr.	20·0	26·5	21·4	6·2	41·4	74·1
	16 ,,	13·6	14·1	—	—	—	—
*p*H 7·2, 25°	30 min.	1·4	85·5	—	—	1·4	86·9
	60 ,,	6·1	83·8	—	—	6·1	89·9
	120 ,,	11·8	76·9	—	—	11·8	88·7
*p*H 8·5, 25°	30 min.	5·2	85·6	—	—	5·2	90·8
	60 ,,	16·5	82·1	—	—	16·5	98·6
	90 ,,	28·3	74·4	—	—	28·3	102·7
	120 ,,	42·3	56·1	Trace included in former		42·3	98·4

Table 4. *Variations in the yield of choleglobin and cholehaemochromogen*

30 min., *p*H 8·5, 37° In % of initial haemoglobin			80 min., *p*H 8·5, 37° In % of initial haemoglobin					
Choleglobin	Haemoglobin	Sum	Choleglobin	Haemoglobin	Cholehaemochromogen	Protohaemochromogen	Total cholehaem	Sum
22·9	80·3	103·2	30·0	49·5	16·5	4·0	46·5	100·1
30·2	72·5	102·7	26·8	46·1	17·0	0·4	43·8	90·3
27·6	69·1	96·7	27·0	53·6	19·3	0·2	46·3	100·0
35·8	69·4	105·2	—	—	—	—	—	—
42·4	69·0	111·4	—	—	—	—	—	—
41·8	60·9	102·7	—	—	—	—	—	—
32·4	67·0	99·4	—	—	—	—	—	—
33·3 ± 2·7	69·7 ± 2·0	103·1	27·9 ± 1·0	49·7 ± 2·2	17·6 ± 0·8	1·5 ± 1·2	45·5 ± 0·9	96·7
				Standard deviation				
7·3	5·4	—	1·8	3·8	1·5	2·1	1·5	—

The reaction velocity of the choleglobin formation at *p*H 8·5 is initially more than double that at *p*H 7·2. The temperature coefficient is rather high (Q_{10} of the initial reaction about 3·5–4·0 between 25° and 37°). The concentration of choleglobin in the solution reaches a maximum, which is higher at 25° than at 37°; after this it remains constant, while more green pigment is formed. At first the latter contains little protohaemochromogen, but later the proportion of the latter increases. Finally the choleglobin concentration begins to decrease. Up to this point there is no indication of by-reactions, the sum of protohaematin and cholehaematin compounds being approximately 100 %. In the later stages, at *p*H 8·5 later than at *p*H 7·2, there is evidently destruction of choleglobin by further oxidation.

Formation of choleglobin by reducing substances other than ascorbic acid

Of other reducing substances reducton is of particular interest. Anderson & Hart [1934] describe the formation of green pigment by glucose+glycine+ammonia in alkaline phosphate buffers, and it is possible that reducton, chemically closely related to ascorbic acid, was the active agent in these mixtures. Crystalline reducton was prepared according to v. Euler & Martius [1933]. It is seen from the data in Table 5 that it undergoes coupled oxidation with haemoglobin and causes as rapid a formation of choleglobin as ascorbic acid. We were, however, unable to find reducton in the mixture used by Anderson & Hart by the indophenol reduction test. Thiolacetic acid also formed choleglobin only slightly less rapidly than did ascorbic acid. Glutathione caused only a very slow reaction, and even this seems to be brought about by impurities. There was considerably less choleglobin formed when glutathione purified over its cadmium salt was used. With cysteine no formation of choleglobin was observed, although a small amount of green precipitate was formed. Both glutathione and cysteine caused a considerable formation of methaemoglobin (cf. Table 6).

Table 5. *Formation of choleglobin by reducing substances other than ascorbic acid*

*p*H 7·2, 37°, haemoglobin 0·7 %, reducing substances in a concentration equivalent to 100 mg. per 100 ml. of ascorbic acid, in the case of thiolacetic acid to 50 mg. per 100 ml. of ascorbic acid.

		In % of initial haemoglobin					
Reducing substances	Incubation time	Choleglobin	Haemoglobin	Cholehaemochromogen	Protohaemochromogen	Total cholehaem	Sum
Ascorbic acid	90 min.	25·4	61·9	18·3	0·3	43·7	105·9
Reducton (crystalline)	90 min.	33·8	54·6	8·4	4·6	42·2	101·4
Thiolacetic acid	90 min.	19·5	71·5	4·2	6·6	23·7	101·8
Glutathione, crystalline	19 hr.	24·4	65·6	5·6	4·8	30·0	Assumed 100
Glutathione, purified over Cd-salt	18½ hr.	6·5	—	Small amount insoluble in NaOH		--	—

Formation of choleglobin in the presence of low concentrations of ascorbic acid

The experiments described hitherto were carried out with ascorbic acid concentrations of 50–100 mg. per 100 ml. In tissue cells the concentration of ascorbic acid is smaller, of the order of 5–50 mg. per 100 ml. (cf. Giroud [1937]). In guinea-pig spleen we found values of 6–15 mg. per 100 ml. with an average of 10·7 mg. per 100 ml.

We have, therefore, carried out some experiments with lower ascorbic acid concentrations. It must be borne in mind that with the comparatively large haemoglobin concentration required for the quantitative study of choleglobin formation, these small amounts of ascorbic acid are rapidly destroyed. In the tissues, however, fresh ascorbic acid can be made available by back-reduction of dehydroascorbic acid with reduced glutathione [Borsook *et al.* 1937; Schultze *et al.* 1937–38; cf. also Genevois & Cayrol, 1939]. Lemberg *et al.* [1938, 1] have shown that reduced glutathione saves ascorbic acid from the oxidation by haemochromogen and does not prevent the oxidation of haemochromogen to verdohaemochromogen. Reduced glutathione accompanies ascorbic acid in the cells of liver and spleen in rather high concentration. In guinea-pig spleen we found 207 mg. reduced glutathione per 100 g. of fresh organ. We have therefore *studied the action of small concentrations of ascorbic acid on oxyhaemoglobin in the absence and in the presence of glutathione on the formation of choleglobin* and the results are given in Table 6.

Table 6. *Formation of choleglobin with low concentrations of ascorbic acid in the presence of glutathione*

0·14 g. of haemoglobin in 20 ml. phosphate buffer *p*H 7·2, 37°

Ascorbic acid mg.	GSH mg.	ϵ_{630} of CO-haemochromogens after 90 min. incubation	Biliverdin after acid splitting of 5 ml.	Spectroscopic observations after 2½ hr. incubation
—	—	0·27	None	Oxyhaemoglobin
—	50	0·39	Trace	Oxyhaemoglobin and methaemoglobin; band 634 mμ disappearing on reduction with $Na_2S_2O_4$
2	—	0·62	Trace	Band at 630 mμ resistant to $Na_2S_2O_4$
2	50	1·05	Considerable amount	Band at 630 mμ resistant to $Na_2S_2O_4$; some green pigment
20	—	2·07	Large amount	Band at 630 mμ resistant to $Na_2S_2O_4$; large amount of green pigment

Even small concentrations (10 mg. per 100 ml.) of ascorbic acid cause a noticeable formation of choleglobin, much larger than that produced by glutathione in a concentration of 250 mg. per 100 ml. In the presence of both glutathione and ascorbic acid more choleglobin is formed than by ascorbic acid alone, and the increase in absorption is significantly larger than can be explained by additive effect. Under the conditions prevailing in the tissue cell haemoglobin must, therefore, undergo oxidation to choleglobin. Actually the condition in the tissue cells is even more favourable than in our experiment, as it will be seen below that low oxygen pressure increases the rate of choleglobin formation while not affecting the destruction of ascorbic acid.

Rate of oxidation of ascorbic acid and effect of oxygen partial pressure on the oxidation of haemoglobin and ascorbic acid

Table 7 gives the percentage losses of ascorbic acid during the incubation of ascorbic acid (100 mg. per 100 ml.) with a 0·7 % haemoglobin solution at 37° and *p*H 7·2 and 8·5; the reaction mixture was kept in flat culture flasks under the conditions previously described.

Table 7. *Ascorbic acid destruction*

Incubation time in min.	% loss of ascorbic acid	
	*p*H 7·2	*p*H 8·5
30	10	26
45	—	—
60	23	—
90	31	45
120	40	—
155	—	55
180	51	—
225	—	74

By comparison with the rate of choleglobin formation given in Table 3 the oxidation ratio—molecules of ascorbic acid oxidized per molecules of haemoglobin oxidized to choleglobin—can be found for the time experiments. This ratio is 10·6 after 30 min. and 11·7 after 80 min. incubation at *p*H 8·5; at *p*H 7·2 the values are 9·6 after 30 min. and 14·0 after 120 min. If 1 mol. O_2 is required to oxidize haemoglobin to choleglobin and 0·5 mol. to oxidize ascorbic acid to dehydroascorbic acid, about one-sixth of the O_2 taken up during the first 30 min. at either *p*H is used for the formation of choleglobin and five-sixths for that of ascorbic acid. Actually somewhat more O_2 will be used for the further oxidation of dehydroascorbic acid.

In order to study the effect of the O_2 partial pressure on the oxidation of haemoglobin and ascorbic acid and on the oxidation ratio under more defined conditions we have carried out experiments in Warburg flasks in which the reaction mixture was shaken with air and with a nitrogen-air mixture containing 2 % O_2. We have shown [Lemberg *et al.* 1938, 2] that in the coupled oxidation of pyridine haemochromogen and ascorbic acid lowering of the O_2 concentration decreases the oxidation ratio from 59 in O_2 to 32 in air and to 12 in a nitrogen-air mixture containing 4 % O_2. The rate of oxidation of pyridine-haemochromogen was little affected, while that of the ascorbic acid oxidation was proportional to the O_2 partial pressure. Table 8 shows that in the coupled oxidation of haemoglobin and ascorbic acid the oxidation ratio is also much lower with low O_2 concentration. Here, however, the rate of oxidation of ascorbic acid is little affected, while that of the choleglobin formation is greatly increased with low O_2 concentrations.

Table 8. *Effect of O_2 pressure on choleglobin formation and ascorbic acid destruction*

Haemoglobin 0·7 %, ascorbic acid 100 mg per 100 ml.; *p*H 7·2, 37°
Total volume of solution: 2·6 ml; in central cup, 0·4 ml KOH.

O_2 concentration	Piperidine dithiocarbamate	Incubation time min	O_2 consumption µmol	Ascorbic acid oxidation µmol.	Choleglobin in % of original haemoglobin	Formation in µmol	Oxidation ratio	(mol. O_2 consumed)/(mol. choleglobin formed)
20 %	Absent	30	2·41	4·06	4·8	0·05	81	**48**
		45	2·90	4·92	9·9	0·10	49	**29**
Standing in air	Absent	30	—	1·48	14·1	0·155	10	—
2 %	Absent	30	2·34	4·52	—		—	—
		45	3·11	5·80	38·1	0·38	15	8·5
20 %	100 µg.	30	4·7		—	—	—	—
		45	6·5	—	23·8	0·23	< 54	28
2 %	100 µg.	30	3·9		—	-	—	—
		45	5·1	—	60	0·58	~ 16	9

Whatever may be the correct explanation of the reaction mechanism (cf. below), it is clear that the low O_2 pressure in the tissue cells favours the oxidation of haemoglobin to choleglobin.

The rate of choleglobin formation in laked erythrocytes

For the experiments described hitherto, solutions of crystalline horse oxyhaemoglobin were used. Table 9 gives the measurements of the velocity of choleglobin formation by coupled oxidation with ascorbic acid under the same conditions as those of Table 3, using, however, laked horse erythrocytes diluted to a haemoglobin concentration of 0·72 %, instead of the solution of oxyhaemoglobin.

Table 9. *Choleglobin formation in laked cells with ascorbic acid*

Conditions	Incubation time in min.	Choleglobin in % of initial haemoglobin: Laked cells	Choleglobin in % of initial haemoglobin: Laked destromatized cells
*p*H 7·2, 37°	15	1·3	5·3
	30	5·1	15·0
*p*H 8·5, 37°	15	22	—
	30	35	—

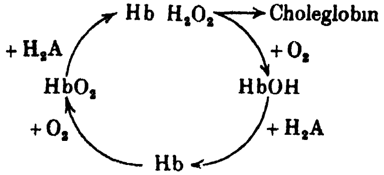
A comparison with the data compiled in Table 3 shows that at *p*H 8·5 the reaction velocity is almost the same, when laked horse cells are used instead of a solution of horse oxyhaemoglobin. At *p*H 7·2, however, choleglobin is formed much more slowly from laked red cells. This indicates the presence in the erythrocytes of a factor inhibiting the formation of choleglobin. This factor is contained in the stromata. When the stromata are removed by keeping the laked cells at a *p*H of 5·8 in the refrigerator and centrifuging, the haemoglobin of the destromatized red cells is now attacked by ascorbic acid and O_2 as rapidly as that in a solution of crystalline oxyhaemoglobin.

These observations help us to explain why there is no, or at least only a very slow, formation of choleglobin in the red cells. From the figures given in Table 6 it can be calculated that, in the presence of 250 mg. per 100 ml. of glutathione, ascorbic acid in a concentration of 10 mg. per 100 ml. is still able to transform 12 % of 0·7 % haemoglobin into choleglobin in 90 min. The concentration of ascorbic acid in the erythrocytes is still considerably smaller. In rabbit erythrocytes we found only 0·35 mg. per 100 ml., considerably less than the values reported by Stephens & Hawley [1936] for human erythrocytes (0·85–1·2 mg. per 100 ml.); in rabbit's plasma are found higher concentrations than in the cells (0·8–1·9 mg. per 100 ml.), while Stephens & Hawley report 1·1–1·35 mg. per 100 ml. for normal human plasma. From our figures it can be concluded that the membrane of the red cell offers some protection to the haemoglobin contained in it by its very incomplete permeability to ascorbic acid [cf. also Borsook *et al.* 1937]. Even so one would still expect a slow, but substantial haemoglobin destruction in the erythrocytes. This is further decreased, if not prevented, by the inhibiting factor in the stroma, which in the cell is probably still much more effective than in the great dilution in which it is present in our dilute laked cell solutions. Whether these factors are able to prevent the formation of choleglobin or other bile pigment-haemoglobins in the red cell completely, is doubtful, and this problem will be discussed in the succeeding paper.

The protection of the plasma ascorbic acid from oxidation by the red cells [Kassan & Roe, 1940] is probably due to this stroma factor rather than to the glutathione of the red cell, with which the plasma ascorbic acid is not in direct contact; Borsook *et al.* [1937] did not find evidence for the reduction of dehydroascorbic acid added to blood. The rapid destruction of ascorbic acid in the blood following haemolysis is due to the coupled oxidation with haemoglobin.

The reaction mechanism

In Part 1 of this series [Lemberg *et al.* 1939] we have shown that the formation of choleglobin by the coupled oxidation of haemoglobin with ascorbic acid cannot be due to hydrogen peroxide formed by autoxidation of ascorbic acid, since the reaction takes place in the presence of copper inhibitors which prevent the autoxidation. Nevertheless, hydrogen peroxide acting on haemoglobin in the presence of ascorbic acid forms choleglobin. We have explained these facts by assuming as first step of the reaction hydrogen-transfer from ascorbic acid to oxyhaemoglobin with the formation of a ferrous haemoglobin-H_2O_2 compound, and have assumed the following cycle in order to explain the destruction of many more ascorbic acid molecules than haemoglobin molecules in the coupled oxidation:



Hb reduced haemoglobin
HbO_2 oxyhaemoglobin
HbOH methaemoglobin
H_2A ascorbic acid

We assume the ferrous haemoglobin-hydrogen peroxide compound to undergo an intramolecular peroxidative reaction, the porphyrin ring being oxidized by the O_2 of the iron-bound hydrogen peroxide of the same molecule.

The reaction mechanism of the formation of verdohaemochromogen from pyridine-haemochromogen by coupled oxidation with ascorbic acid was explained in a similar way [Lemberg *et al.* 1938, 1, 2]. We have found that hydrogen peroxide acting on pyridine-haemochromogen in the presence of ascorbic acid and in the absence of O_2 leads to a precursor of verdohaemochromogen [Lemberg *et al.* 1938, 2]. The green compound obtained by Haurowitz [1937] by reduction of the ferric pyridine haemochromogen-hydrogen peroxide compound by pyrogallol is also verdohaemochromogen.

Hydrogen peroxide forms choleglobin from haemoglobin in the presence of ascorbic acid. We have now found that even in the absence of reducing substances hydrogen peroxide causes a substantial formation of choleglobin from reduced haemoglobin, while much less is formed from oxyhaemoglobin. To 3·5 ml. of an 0·7 % haemoglobin solution, reduced by evacuation under nitrogen, 0·5 ml. of 0·6 % hydrogen peroxide was added under nitrogen. After 2 min. at 20° the excess hydrogen peroxide was destroyed by a drop of catalase solution and the choleglobin content determined spectrophotometrically. It was found that 16 % of the reduced haemoglobin had become transformed into choleglobin. If oxyhaemoglobin reacted with hydrogen peroxide under the same conditions but in air, only 7 % was transformed into choleglobin. Methaemoglobin yielded 6 % choleglobin under nitrogen. Hydrogen peroxide is evidently able to reduce some methaemoglobin to haemoglobin in the same way as catalase is reduced to its ferrous form by hydrogen peroxide [Keilin & Hartree, 1937].

These observations show that the effect of the O_2 pressure on the oxidation ratio (cf. above and Table 8) can be understood as autoxidation of the ferrous haemoglobin-H_2O_2 compound competing with its transformation to choleglobin. The methaemoglobin-hydrogen peroxide compound is known to be unstable and to give methaemoglobin. The fact that high O_2 pressure lowers the oxidation ratio cannot be explained by the assumption that the autoxidation of ascorbic acid competes with its coupled oxidation with haemoglobin, since even at high O_2 pressure copper inhibitor does not diminish the oxidation ratio and the effect of O_2 pressure in the oxidation ratio in the presence of copper inhibitor is the same as in its absence.

We have ascertained that methaemoglobin can be an intermediate product in the cycle. It reacts with about the same velocity as oxyhaemoglobin, and the effect of O_2 pressure on the ratio 'mol. of O_2 consumed to mol. of

Table 10. *Choleglobin formation from methaemoglobin*

Methaemoglobin 0·7 %; ascorbic acid 100 mg. per 100 ml.; *p*H 7·2; 37°.
Volume of solution: 2·6 ml.; in central cup, 0·4 ml. KOH.

O_2 concentration	Incubation time min.	O_2 uptake in μmol.	Choleglobin formation: In % of original haemoglobin	Choleglobin formation: In μmol.	Ratio (mol. O_2 consumed)/(mol. choleglobin formed)
20 %	1	0·36	—	—	—
	15	1·69	—	—	—
	30	2·59	—	—	—
	45	3·35	8·4	0·094	36
2 %	1	0·13	—	—	—
	15	1·20	—		—
	30	2·00	—	—	—
	45	2·90	24·6	0·276	10·5

choleglobin formed' is the same as with oxyhaemoglobin (compare Table 10 with Table 8).

While the effects of the O_2 pressure on the oxidation ratios in choleglobin and verdohaemochromogen formation and on the reaction velocities of verdohaemochromogen formation and destruction of ascorbic acid in it can be understood by the notion that the autoxidizable ferrous haem compounds undergo oxidation of their prosthetic groups, it still remains unexplained why the optimum of the rate of choleglobin formation is at low O_2 pressure (cf. Table 8), while the rate of verdohaemochromogen formation does not depend on O_2. Since the effects of the O_2 pressure on the oxidation of ascorbic acid also differ in both cases in an analogous manner (a high O_2 pressure favouring the destruction of ascorbic acid in the coupled oxidation with pyridine-haemochromogen, but no influence of O_2 pressure on ascorbic acid oxidation with haemoglobin), it is likely that an explanation of the phenomenon must be concerned with the reaction initiating the cycle, the reaction of oxyhaemoglobin with ascorbic acid. There is a remarkable parallel between choleglobin and methaemoglobin formations from oxyhaemoglobin, the rates of both reactions having maxima at low O_2 pressures in spite of the fact that the reactions require O_2. The range in which this optimum is found, is one in which there is partial dissociation of oxyhaemoglobin. The observations of Brooks [1935] make it likely that the explanation has to be sought in an intermediate between Hb_4 and Hb_4O_8, e.g. Hb_4O_2, being the substance converted into methaemoglobin. The same applies probably to choleglobin formation. Brooks himself rejects this explanation on the basis of calculations involving various constants which hitherto have not been established securely. His own explanation is, however, quite unsatisfactory. Besides assuming an inhibition by O_2 of obscure nature, he believes that the rate of methaemoglobin formation is proportional to the concentration of reduced haemoglobin which is decreased by high O_2 pressure. Brooks overlooks the fact that the concentration of reduced haemoglobin is low at high O_2 pressure, because it reacts more rapidly with O_2. There is no reason to assume that this reaction should lead to oxyhaemoglobin exclusively, while another reaction of reduced haemoglobin with O_2 would yield methaemoglobin.

DISCUSSION

In a previous paper [Lemberg *et al.* 1938, 1] we had put forward the hypothesis that dehydroascorbic acid, formed by the coupled oxidation of haemoglobin and ascorbic acid during the bile pigment formation *in vivo*, is reduced back to ascorbic acid by reduced glutathione, and that oxidized glutathione in turn is reduced by the glucose dehydrogenase system. The latter assumption was based on the experiments of Mann [1932]. Meanwhile Schultze *et al.* [1939] have shown that neither ascorbic acid nor glutathione nor a mixture of the two substances can act as a hydrogen-carrying system between nicotine-haemochromogen and the glucose dehydrogenase system or coenzyme. This result scarcely affects our hypothesis that the ascorbic acid-glutathione system plays an important part in the physiological destruction of haemoglobin, since it is not of importance how the glutathione is restored. What principally matters is that ascorbic acid and haemoglobin undergo coupled oxidation under the conditions found in the cells of those organs in which bile pigment formation takes place; for this we bring forward evidence in the present paper. Secondly, ascorbic acid must be somehow restored, because the daily haemoglobin destruction involves the oxidation of a larger amount of ascorbic acid than is replaced in the food. The fact that dehydroascorbic acid has antiscorbutic activity makes it almost certain that it can be reduced *in vivo*. Several authors (cf. above) have shown that reduced glutathione is able to reduce dehydroascorbic acid in liver cells as well as *in vivo*, but recently Schultze *et al.* [1937–38] have thrown some doubts on the actual occurrence of this reaction *in vivo*. We agree with those authors that their results make it unlikely that the ascorbic acid-glutathione system plays a major role in cell respiration [cf. King, 1939]. A sweeping generalization that the reduction of dehydroascorbic acid by glutathione does not occur in the animal body would, however, not be justified. The experiments of Schultze *et al.* [1937–38, p. 403] were carried out with a proportion of glutathione to ascorbic acid which was much lower than that actually found in liver and spleen, and even so they showed only that the reduction of dehydroascorbic acid by glutathione was not complete.

Other reducing systems may play a role in the physiological oxidation of haemoglobin, but so far the ascorbic acid-glutathione system is the only one known to occur in cells that is able to catalyse the formation of choleglobin under physiological conditions.

SUMMARY

A spectrophotometric method has been worked out for the determination of the choleglobin content in mixtures of choleglobin and haemoglobin, and thus the measurement of the rate of choleglobin formation.

The spectrophotometric investigation indicates that in the initial stages of the reaction choleglobin is the only product of oxidation of haemoglobin, while later other products are formed in addition to choleglobin and choleaemochromogen.

The initial reaction velocity of choleglobin formation is about twice as large at pH 8·5 as it is at pH 7·2. The temperature coefficient is high ($Q_{10} = 3{\cdot}5 - 4$ between 25 and 37°).

Reducton forms choleglobin as fast as ascorbic acid, thiolacetic acid more slowly, glutathione and cysteine very slowly, if at all.

Choleglobin is rapidly formed from haemoglobin under physiological conditions (pH 7·2, 37°) and with concentrations of glutathione and ascorbic acid such as occur in tissue cells, glutathione increasing the rate of choleglobin formation more than can be due to a merely additive effect.

At low O_2 pressure (15 mm.) the formation of choleglobin proceeds faster than in air, while the oxidation of ascorbic acid is little affected. The same is found in experiments carried out in the presence of copper inhibitor which prevents the

autoxidation of ascorbic acid. The theoretical implications of these observations are discussed. In air, without shaking, about 10 molecules of ascorbic acid are oxidized per molecule of choleglobin formed.

In the red cells haemoglobin is protected from rapid oxidation by the small permeability of the cell membrane to ascorbic acid and by an inhibiting factor in the stromata.

REFERENCES

Anderson & Hart (1934). *J. Path. Bact.* **39**, 465.
Borsook, Davenport, Jeffreys & Warner (1937). *J. biol. Chem.* **117**, 237.
Brooks (1935). *Proc. roy. Soc.* B, **118**, 560.
v. Euler & Martius (1933). *Liebigs Ann.* **505**, 73.
Genevois & Cayrol (1939). *Enzymologia*, **6**, 352.
Giroud (1937). *Ergebn. Vitamin. Hormonforschung*, **1**, 68.
Haurowitz (1937). *Enzymologia*, **4**, 139.
Kassan & Roe (1940). *J. biol. Chem.* **133**, 579.
Keilin & Hartree (1937). *Proc. roy. Soc.* B, **124**, 397.
King (1939). *Symposia on Quantitative Biology, Cold Spr. Harb.* **7**, 137.
Lemberg, Cortis-Jones & Norrie (1938, 1). *Biochem. J.* **32**, 149.
—— —— —— (1938, 2). *Biochem. J.* **32**, 171.
—— Legge & Lockwood (1939). *Biochem. J.* **33**, 754.
Mann (1932). *Biochem. J.* **26**, 785.
Schultze, Harrer & King (1939). *J. biol. Chem.* **131**, 5.
—— Stotz & King (1937–38). *J. biol. Chem.* **122**, 395.
Stephens & Hawley (1936). *J. biol. Chem.* **115**, 653.

35. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN

4. THE 'LABILE IRON' IN BLOOD AND ITS INCREASE DURING CHOLEGLOBIN FORMATION[1]

By J. W. LEGGE and R. LEMBERG

From the Institute of Medical Research, Royal North Shore Hospital, Sydney

(*Received 3 March 1941*)

It has been shown by Lemberg [1935] and Lemberg & Wyndham [1937] that the iron of a bile pigment-haematin compound (verdohaemochromogen) is more easily detached by acids than haemoglobin iron. If choleglobin is a bile pigment-haemoglobin it is to be expected that its formation during the coupled oxidation of haemoglobin and ascorbic acid will be accompanied by an increase in the 'labile iron'.

Barkan and his collaborators have shown in several papers that a small part of the iron in erythrocytes is split off more readily by acids than that of the remainder, and that 'leicht abspaltbares Bluteisen', for which we shall write in short ESI, accompanies oxyhaemoglobin through its purification and recrystallization. The initial values of ESI have, therefore, to be taken into account in the study of ESI in choleglobin solutions. According to Barkan & Schales [1937; 1938] the source of ESI is a bile pigment-haemoglobin ('pseudohaemoglobin') accompanying haemoglobin in the red cells, and inseparable from it on account of the identity of the protein parts (native globin). If this be correct, pseudohaemoglobin and choleglobin must be identical or very closely related. We have therefore reinvestigated the problem of the ESI in erythrocytes. While our experimental results agree with those of Barkan, except in one or two points, we arrive at different conclusions as to the explanation of these observations.

By this investigation we have also established the values of the initial ESI obtained from haemoglobin solutions in the presence of ascorbic acid in such concentrations as were used for the coupled oxidation. Thus we are enabled to measure the increase of ESI occurring during this reaction and to correlate it with the increase of choleglobin concentration, estimated spectrophotometrically with the method described in the preceding paper.

EXPERIMENTAL

Methods

Determination of ESI. Barkan incubates the diluted solution of haemoglobin with 0·4 % HCl for 16–24 hr., and then obtains by ultrafiltration a protein-free solution, in which he estimates the iron by a ferric thiocyanate method.

We found that our ultrafiltrates through Zsigmondy filters ('Cella finest' and 'Ultrafine') contained some protein breakdown products. These came out of solution at the *p*H used for the colorimetric estimation of iron as the ferrous $\alpha\alpha'$-dipyridyl complex in the method of Hill after Shorland & Wall [1936]. In

---

[1] The work was carried out under a grant from the National Health and Medical Research Council of Australia.

order to use this method we prepared protein-free filtrates by trichloroacetic precipitation after incubation with HCl; blanks were, of course, carried out and the blank values subtracted. The results did not differ from those obtained with the Barkan method. A choleglobin solution, which according to the latter contained 10·25 % and in the presence of CO 11·3 % ESI, gave the values 11·0 and 10·25 % with our method. Barkan [1937] has also made use of trichloroacetic acid precipitation and confirmed earlier results of other workers that it gives the same results as the rather tedious ultrafiltration. He claims that in the presence of CO too low values of ESI in blood are occasionally found with the trichloroacetic acid method, so that the CO inhibition of the removal of ESI appears too high. The CO inhibitions that we observed in blood were, however, the same as those found by Barkan.

As a routine method we adopted the following procedure. To 1 ml. of the solution to be determined (blood diluted 10 times or choleglobin solution prepared from 0·7 % haemoglobin undiluted) was added 1 ml. of iron-free 0·8 % HCl; after mixing the solution was incubated overnight (16 hr.) at 37°. 1 ml. of 7 % trichloroacetic acid was then added, the mixture well shaken and the precipitate filtered off on a Whatman filter No. 42. The iron in an aliquot of the filtrate was then estimated by the method of Shorland & Wall.

If the acid treatment had to be carried out in CO or N_2, the solution was placed in the bottom part of a Thunberg tube and the HCl in the bent stopper. The CO was kept standing over alkaline pyrogallol solution, while the N_2 was freed from O_2 over heated copper asbestos.

Determination of total iron. A suitable aliquot of the solution was taken to dryness in a 6 in. × $\frac{5}{8}$ in. pyrex test tube. After ashing with 0·4 ml. conc. H_2SO_4 and 0·1 ml. perchloric acid (Merck) the solution was cooled and either made up to 10 ml. with glass-distilled water and analysed by the dipyridyl method; or after 2–3-fold dilution with water the solution was made alkaline with 5*N* NH_4OH containing ammonium thiolacetate. During this procedure it was cooled with ice water. After keeping for 5–10 min. with repeated shaking in order to keep the complex in the ferric state, the solution was compared colorimetrically with an iron standard treated in the same way. The two methods gave identical results.

The blood ESI and its behaviour towards CO and reducing substances

Thirteen determinations of the ESI on seven samples of human blood exposed to air (ESI_{O_2}) gave results which varied from 3·6 to 7 % of the total iron, the mean being 5·35 % and the standard deviation ± 1·17 %. Thirteen determinations on the same batch of crystalline horse oxyhaemoglobin gave values ranging from 3·5 to 11 %, with a mean of 6·9 % and a standard deviation of ± 2·5 %. During the preparation of crystalline horse oxyhaemoglobin the ESI_{O_2} varied from 4·9 % in the cells to 5·4, 5·7 % in the first crystallization, 4·0, 3·5 % in the second and 8·5, 9·8 % in the third. Recrystallization thus did not alter the ESI_{O_2} significantly. These values agree with those found by Barkan and other observers.

Effect of carbon monoxide. We can also confirm that under CO much less iron is detached than in air. The ESI observed under CO (ESI_{CO}) is only about one-third of that observed when the splitting is carried out in air (ESI_{O_2}). The average amounts of ESI_{CO} as % of ESI_{O_2} in thirteen estimations on crystalline horse oxyhaemoglobin varied from 21 to 47 with a mean of 31 and a standard deviation of ± 5. Determinations on sheep blood came within this range. This would mean an inhibition of the detachment of iron by CO of 69 % on the average.

Effect of reducing substances and inert gases. Barkan & Berger [1928] have already found that H_2 has an effect similar to that of CO.

Table 1 shows the effect of evacuation and addition of reducing substances ($Na_2S_2O_4$ and ascorbic acid) on the detachment of iron. These experiments were carried out with both crystalline horse oxyhaemoglobin and laked sheep cells. No difference was observed, so they are given together. Evacuation and ascorbic acid in concentrations of 30 mg. per 100 ml. or more decrease the detachment of iron by acids to the same degree as CO or H_2. The amount split off in presence of $Na_2S_2O_4$ is a little larger, because the SO_2 developed on acidification of the solutions removes some iron from haemoglobin.

Table 1. *Influence of evacuation and reducing agents on blood ESI*

Conditions	ESI found in % of ESI_{O_2}	No. of experiments averaged
In vacuo	33	2
Added $Na_2S_2O_4$, 50 μg per ml.	89	2
„ 100 „	51	5
„ 300 „	46	8
„ excess	53	4
Added ascorbic acid, 50 μg. per ml.	80	2
„ 100 „	80	6
„ 300 „	38	6
„ 1200 „	25	2

Barkan & Schales [1937] claim that by the addition of small amounts of $Na_2S_2O_4$ the inhibition of the iron detachment by CO can be further increased, and base important conclusions on this observation. While the theoretical side of the question will be discussed later, we point out here that the experiments of Barkan & Schales lend only slender support to this claim. The authors report three experiments in which the ESI_{CO} was further lowered by addition of 130–330 μg. $Na_2S_2O_4$ per ml. Examination of their figures reveals, however, that in two of these experiments the amount split under CO in the absence of $Na_2S_2O_4$ was abnormally high (ESI_{CO} still 75 % and 58 % of ESI_{O_2}, instead of the usual 31 %); and that ESI_{CO} in the presence of the reducer (25 % of ESI_{O_2}) was not significantly smaller than usually without reducer and still inside the range of the figures for ESI_{CO} in percentage of ESI_{O_2} (21–47 % in our experiments). It is very likely that the saturation of these solutions with CO was somehow incomplete and that the effect of the reducer was only to bring it to completion. In one experiment only ESI_{CO} was 33 % of ESI_{O_2} without reducer and was further decreased by the addition of $Na_2S_2O_4$ to 14 %. Occasional stray results do, however, occur and cannot be considered as sufficient evidence.

We have therefore repeated these experiments using various concentrations of $Na_2S_2O_4$ and also of ascorbic acid added to solutions of crystalline horse oxyhaemoglobin (Table 2).

Table 2. *Influence of reducing agents on blood ESI in the presence of CO*

Conditions	ESI_{CO} in % of ESI_{O_2}
Without reducer, CO	28
Added $Na_2S_2O_4$, 130 μg. per ml.	14; 31
„ 300 „	31
Added ascorbic acid, 50 μg. per ml.	32
„ 120 „	15; 29
„ 300 „	28
„ 1200 „	24

Again a decrease of the iron split off was only observed in two experiments out of eight, and in each case repetition of the same experiment failed to confirm the low value. The decrease cannot therefore be considered as significant.

Experiments on the nature of the compound from which the blood ESI is derived

In the first paper of this series [Lemberg, Legge & Lockwood, 1939] we have already mentioned that CO does not decrease the amount of ESI liberated from choleglobin solutions, and have adduced this as evidence against the identity of choleglobin and the substance in blood which is the source of the ESI. The choleglobin solutions contain, however, ascorbic acid in concentrations between 50 and 100 mg. per 100 ml. and from Tables 1 and 2 it is evident that the blood ESI would also not be decreased much further by CO in the presence of ascorbic acid in this concentration. After removal of ascorbic acid from choleglobin solutions by prolonged dialysis CO has still no effect on the yield of ESI, but during dialysis oxycholeglobin, if present, may be transformed into ferric choleglobin and therefore not react with CO. It cannot be absolutely excluded, therefore, that CO may inhibit the removal of iron from an oxycholeglobin with reversibly bound O_2, the existence of which is not proved. It will be seen from the discussion below, that this point is no longer of importance, since we believe that the CO inhibition of the detachment of ESI in the blood has to be explained in a totally different way.

We have also briefly described spectroscopic observations which made us doubt the occurrence of choleglobin in red cells. They are given here in greater detail. When a strong solution of oxyhaemoglobin or of laked erythrocytes is treated with $Na_2S_2O_4$ in presence of air, the band of choleglobin is observed, and that of cholehaemochromogen when the haemochromogen is formed by pyridine or alkali and $Na_2S_2O_4$ without precautions. When, however, reduced haemoglobin is formed by evacuation or when the reductions with $Na_2S_2O_4$ are carried out in a medium free from O_2, no trace of these bands can be observed spectroscopically. In the first-mentioned experiments choleglobin is, therefore, an artefact, doubtless formed from haemoglobin by the action of traces of hydrogen peroxide, which is well known to arise by the action of O_2 on $Na_2S_2O_4$ solutions. While these experiments show once more the ease with which choleglobin arises from haemoglobin, they do not offer any evidence for its occurrence in red cells. In the absence of O_2 another absorption band was observed in solutions of reduced haemoglobin and in denatured globin and pyridine haemochromogens, lying at about 660 mμ. This band disappeared on oxygenation and reappeared on reduction. It was not observed in solutions of CO-haemoglobin or in laked blood treated with coal gas with or without addition of $Na_2S_2O_4$. This compound may be a source of some of the blood ESI.

From the spectrophotometric curves of CO-haemochromogen prepared from laked blood or crystalline oxyhaemoglobin (Fig. 1 of Part 3) we can deduce that choleglobin iron cannot form as much as 5 % of the total iron. There is only a very slight hump of the absorption curve at 630 mμ indicating less than 1 % of choleglobin. Even if pure CO-protohaemochromogen had zero absorption at 630 mμ, which from the form of the absorption curve is very unlikely, the choleglobin content could not be higher than 3·9 %.

We have already briefly reported that the treatment of laked erythrocytes and of horse oxyhaemoglobin with acid yields, indeed, some bile pigment, and quantitative estimations will be given in the succeeding paper. While this proves the occurrence of some bile pigment haemoglobin in red cells, the bile pigment yield was much lower than that expected from a bile pigment haemoglobin content of 5 % which ought to be present if all ESI were derived from it.

We have therefore investigated whether some of the ESI may not originate from haemoglobin. The arguments which Barkan brings forward against this assumption will be discussed below, while here we present a few experiments. We have tried whether methaemoglobin synthesized in a way which excludes the presence of bile pigment-iron compounds, does not yield any ESI. Pure crystalline haemin should not, according to the way it is prepared, contain any bile pigment haematin; we have further made sure of this by extracting its ether-pyridine solution repeatedly with 20 % HCl. After washing with water the precipitated haemin was dissolved in $N/10$ ammonia and coupled at once with the optimal amount of a native horse globin solution, the iron content of which was known. The alkaline haematin solution must not be allowed to stand, since we have observed that it is not very stable and after a few hours a band may be found at about 630 mμ after addition of native globin and $Na_2S_2O_4$. The synthesized methaemoglobin yielded even slightly more ESI (6·5–9·6 %) than oxyhaemoglobin from red cells (Table 3). Inhibition by CO was not found, nor was it expected since the iron is in the ferric state. The experiment shows that the ESI of the blood may, at least partly, be derived from haemoglobin.

Table 3. *ESI in synthesized methaemoglobin*

The iron contained in the native globin solution (0·50 μg. per ml.) has been subtracted from the ESI values found.

	Total iron	ESI_{O_2}	ESI_{CO}	ESI_{N_2}
μg. per ml.	7·7	0·71; 0·56	0·74	0·50

Finally we have investigated the claims of Barkan & Berger [1930] that the CO-inhibition of the detachment of iron in mixtures of CO-haemoglobin and oxyhaemoglobin is much larger than it ought to be, if it were proportional to the saturation of haemoglobin with CO, and that the CO inhibition persists even after the reconversion of CO-haemoglobin into oxyhaemoglobin. This, indeed, would prove that the CO causing the inhibition of the iron splitting is bound to a compound different from haemoglobin. The experiments of these authors are, however, open to criticism. They claim to get the same results whether they add a known amount of CO dissolved in water to a solution of oxyhaemoglobin, or mix solutions of oxyhaemoglobin and CO-haemoglobin (saturated with CO) of about 1·5 % haemoglobin concentration (blood diluted ten times) in the required proportions. This cannot be correct, since 100 ml. of 1·5 % CO-haemoglobin solution contain 2 ml. of CO bound to haemoglobin, but at 15° at least 2·5 ml. CO in physical solution, and probably more, since this figure is based on the solubility in water, and that in protein solution is likely to be higher. The CO-haemoglobin content in the mixtures was not determined, and it is obvious that the mixtures must have contained a much greater proportion of CO-haemoglobin than Barkan & Berger assumed from the proportion in which the solutions had been mixed. There is, therefore, no evidence that the presumptive disproportionality between the degree of CO-inhibition of the iron splitting and the CO-haemoglobin concentration really exists. We can also not confirm the second claim. While the authors still find a considerable CO-inhibition of the iron detachment after 48 hr. of vigorous aeration of a CO-haemoglobin solution, we found that after 24 hr. aeration the ESI had returned to its original value.

The increase of ESI during choleglobin formation

During the coupled oxidation of haemoglobin and ascorbic acid the ESI of the solution rises simultaneously with the increase in absorption at 630 mμ due to choleglobin. In a series of experiments we have measured the ESI of choleglobin solutions and have compared the increase in ESI with the choleglobin concentration estimated spectrophotometrically as described in Part 3.

The choleglobin solutions contained 50–100 mg. ascorbic acid per 100 ml. From the results reported above it can be deduced that haemoglobin solutions containing ascorbic acid in this concentration yield ESI of about 1·5 % of the total iron. This value was therefore subtracted from the ESI found in our choleglobin solutions. The values thus obtained are given as ΔESI in Table 4. The procedure of subtracting the initial ESI is justified, even if the ESI or part of it were attributable to choleglobin, since the average absorption of CO-protohaemochromogen at 630 mμ has also been subtracted from the absorption values at 630 mμ for the estimation of the choleglobin content.

The values of choleglobin (including cholehaemochromogen in the 80 min. experiments at 37° in Table 4 are in percentage of initial haemoglobin and those of ΔESI in percentage of total iron, while in the bottom row (green pigment) the first figure means cholehaemochromogen in percentage of total haemochromogen (proto + chole) and the second figure ESI in percentage of total iron.

Table 4. *ESI from choleglobin*

Conditions of experiment	Time of incubation min.	Choleglobin	ΔESI	Ratio $\frac{\Delta \text{ESI}}{\text{choleglobin}}$
25°, *p*H 8·5	90	28·3	23·9	0·84
	120	42·3	28·4	0·67
37°, *p*H 8·5	15	17·9	11·7	0·65
	30	33·3	19·5	0·59
	80	45·5	23·8	0·52
37°, *p*H 7·2	30	14·1	12·2	0·86
	80	34·6	21·5	0·62
Green pigment	—	77·3	51	0·66
				Average 0·66

The increase of ESI under various conditions is evidently proportional to the amount of choleglobin formed, but the yield of ESI is only 66 % of that expected theoretically from the amount of choleglobin. The reason for this discrepancy is that a part of the cholehaematin remains unsplit and is still contained as such in the precipitate of denatured protein. If this precipitate is carefully washed free from trichloroacetic acid and redissolved in *N*/10 NaOH, a spectrophotometric determination of the absorption at 630 mμ of the CO-haemochromogen shows that 31 % of the cholehaematin has remained unsplit in this precipitate. It is possible that this observation means that choleglobin is not homogeneous and consists of two compounds, one easily split by acids and one that is not easily split, but the incomplete splitting cannot be considered as definite evidence against uniformity. We have observed similar incomplete splittings with verdohaemochromogen [Lemberg & Wyndham, 1937].

CO does not inhibit the detachment of iron from choleglobin solutions. In a few experiments a slight inhibition by CO (10–20 %) was found, but this is within the experimental range of error and in other experiments ESI_{CO} was found higher than ESI_{O_2}; usually both were equal (e.g. ESI_{O_2} 14·8 μg. per ml.,

ESI_{CO} 15·8, ESI_{CO} in presence of $Na_2S_2O_4$ 15·6). Even after the ascorbic acid had been removed by prolonged dialysis CO had no effect (ESI_{O_2} 18·9 % of the total iron, ESI_{CO} 19·4 %, ESI_{CO} in presence of $Na_2S_2O_4$ 18·4 %). A solution of cyanmetacholeglobin (cf. Part 2) yielded 8·9 % ESI_{O_2}, 7·3 % ESI_{CO} and in presence of $Na_2S_2O_4$ without CO 9·6 %, with CO 10·0 % ESI, all in percentage of total iron. All these differences lie within the range of experimental error.

DISCUSSION

From their observation that CO decreases the yield of labile iron from laked erythrocytes or oxyhaemoglobin solutions to about one-third of its value, Barkan & Schales have concluded that the ESI arises from a bile pigment-haemoglobin (pseudohaemoglobin). They assume that the iron detached by acid splitting in air, but not in CO, i.e. $ESI_{O_2}-ESI_{CO}$ (about 65 % of the ESI and 3·5 % of the total iron), is derived from a bile pigment-oxyhaemoglobin (EO_2), EO_2 being easily split by acids in contradistinction to the CO compound (ECO). The iron that is detached even in the presence of CO (ESI_{CO}), about 35 % of ESI and 1·5 % of the total iron, they believe to be the ferric form of the same bile pigment haemoglobin (E′), since they observed that it can be partially converted into the CO-inhibitable form by reduction with $Na_2S_2O_4$.

In our experiments described above we have been able to confirm the greater part of Barkan's experiments with the exception of the presumptive conversion of E′ into ECO by reduction and CO, for which we do not find conclusive evidence, and their observation that the CO inhibition of the detachment of iron occurs under conditions in which haemoglobin is no longer combined with CO.

We have shown that a small yield of biliverdin can indeed be obtained from erythrocytes and haemoglobin solutions by treatment with acids, and this lends support to the view that at least part of the ESI is attributable to a bile pigment-haemoglobin. The quantitative experiments described in Part 5 indicate, however, that the concentration of this bile pigment-haemoglobin in blood or haemoglobin preparations is too small to account for more than a small part of the ESI.

There are in fact, considerable difficulties in accepting Barkan's deductions about the nature of the ESI. The effect of CO on ESI is not specific, and Barkan explains the decreasing effect of inert gases on the yield of ESI by the assumption that only EO_2 (oxypseudohaemoglobin) and E′ (pseudomethaemoglobin) undergo splitting, while the iron in E (reduced pseudohaemoglobin) as well as in ECO (carboxypseudohaemoglobin) is resistant to acid. For chemical reasons this does not appear likely. Experience with haemoglobin derivatives shows that CO-haemoglobin and reduced haemoglobin are much more readily split than oxyhaemoglobin or methaemoglobin. It is not impossible that the properties of pseudohaemoglobin derivatives are exactly opposite to those of haemoglobin derivatives. Experience with known bile pigment-haematin compounds, e.g. verdohaemochromogen, shows that the ferric and ferrous forms are both split by acid, and that CO, while combining, does not prevent the splitting (cf. Part 5). Choleglobin appears to behave in the same manner.

Barkan & Berger [1928] describe an experiment, in which they acidified the haemoglobin under hydrogen and then aerated the solution. The amount of ESI found under these conditions was not larger than without aeration (equal to ESI_{CO}). In order to explain this result the authors are forced to make the unlikely assumption that E does not unite with O_2 in acid solution. In the presence of acid, E must be split to pseudohaem and no acid haem has been observed that is not readily autoxidizable to acid haematin, whether in the class *of porphyrin-haematins or bile pigment-haematins. The final product obtained under the conditions of the experiment ought to be, therefore, the same as from* EO_2 and E', and the ESI found ought to be equal to ESI_{O_2}, not to ESI_{CO}.

Several authors [Taylor & Coryell, 1938; Ammundsen, 1939] have recently confirmed earlier observations of Klumpp [1935] that a difference of about 5% exists between the O_2 capacity of blood and its CO capacity in the presence of $Na_2S_2O_4$. This indicates the presence of ferric haemoglobin or ferric bile pigment-haemoglobin in normal blood. From magnetochemical observations Taylor & Coryell also conclude that the substance combining with CO in the presence of $Na_2S_2O_4$ but not combining with O_2 is a ferric compound. Barkan & Schales [1939] mention the results of Ammundsen as being explained by the presence of 'pseudohaemoglobin', but this is evidently only partly true. While E + E' form 5% of the total blood iron, only E' (1·5%) combines with CO in presence of $Na_2S_2O_4$ and not with O_2, E (3·5%) would combine with both and thus not contribute to the difference of the gas capacities. The existence of this difference, therefore, supports only the assumption that E' is the ferric form of a bile pigment-haemoglobin, and again E can explain only a fraction of the difference found between the O_2 and CO capacities.

The whole problem is evidently far from being satisfactorily solved, and we offer, therefore, an alternative explanation which, while not fully satisfactory in all respects, appears to us more likely and in better agreement with the observed facts. We shall discuss separately the CO-inhibitable part of the ESI ($ESI_{O_2} - ESI_{CO}$, attributed by Barkan to EO_2) and the CO non-inhibitable part (ESI_{CO}, attributed by Barkan to E), since we do not believe that there is evidence for a chemical relationship of the two forms. There is no evidence that the effect of CO on the blood ESI is specific and that CO acts in a way different from any other inert gas. The experiments indicate solely that presence of O_2 is necessary for setting free of the iron from E. The simplest explanation appears to us to be that the CO-inhibitable iron fraction ($ESI_{O_2} - ESI_{CO}$) is formed from oxyhaemoglobin by an oxidation process during the action of acid on haemoglobin. Recently several authors have observed that the O_2 set free from oxyhaemoglobin by denaturation has strongly oxidative power. Holden [1936; 1937] and Rawlinson [1939] have shown that the globin part of oxyhaemoglobin undergoes oxidation during the denaturation. Lemberg & Legge [1938] found that the O_2 set free from oxyhaemoglobin by acids oxidizes ascorbic acid. In Part 5 it will be shown that under the same circumstances biliverdin is oxidized to biliviolin. Engel [1939] has recently observed that some plasma bilirubin is oxidized during the precipitation of oxyhaemoglobin by alcohol, and that it can be protected against this oxidation by ascorbic acid. In the same way ascorbic acid protects biliverdin from being oxidized during the acid denaturation of oxyhaemoglobin. It is only necessary to assume that the oxidative effect can be turned against the prosthetic group of the haemoglobin molecule as well as towards the globin part. It has already been suggested by Lintzel & Radeff [1928] that ESI is derived from haemoglobin and that the prosthetic group is partially destroyed by the action of acid; the authors' claim is based on their observation that the colour of the acid haematin derived from oxyhaemoglobin is several per cent less in intensity than from the same amount of carboxyhaemoglobin. Barkan & Berger [1930] have accumulated material against this hypothesis, but their arguments are not fully convincing, although Lintzel & Radeff's observation may be explained by a different colloidal state of the 'acid haematins' derived from oxyhaemoglobin and CO-haemoglobin. Some of Barkan's arguments, e.g. the fact that the detachment of ESI is irreversible though incomplete, and the lack of formation or porphyrin, do not speak against our hypothesis. His experiments with mixtures of CO-haemoglobin and oxyhaemoglobin are open to the criticism levelled above against his method of determining the ratio of the two compounds. It is doubtful, whether the slight increase of the ESI percentage in haemoglobin after partial adsorption on alumina, observed by Barkan [1936], is actually due to a preferential adsorption of haemoglobin on alumina; if one remembers the ease with which small amounts of choleglobin are obtained from haemoglobin, it is quite likely that a slight oxidation of haemoglobin to choleglobin occurs during the adsorption experiments; Barkan has omitted to show that the haemoglobin recovered from the alumina contained less ESI.

The majority of Barkan's and our observations can be explained satisfactorily on the assumption that ESI is derived from haemoglobin by oxidation during the acidification. Inert gases or CO would prevent this reaction by transforming oxyhaemoglobin into reduced haemoglobin or CO-haemoglobin, while ascorbic acid would prevent the oxidative removal of iron from the haemoglobin molecule by being oxidized instead, in the same way as it protects added bile pigment. Once the acid denaturation has taken place under exclusion of O_2, aeration would no longer cause oxidative removal of iron, in agreement with the experiment of Barkan & Berger mentioned above. The transformation of oxyhaemoglobin into methaemoglobin does not decrease the yield of ESI [Barkan & Berger, 1928], since the oxidative reaction setting free the iron would occur during the methaemoglobin formation.

One observation in our experiments would still remain unexplained, namely the presence of the usual percentage of ESI in solutions of synthetic methaemoglobin, but this observation too speaks against the theory of Barkan and favours the assumption that some ESI is derived from haemoglobin itself, since any bile pigment-iron compounds have been excluded by the method of preparation.

While the greater part of ESI, about two-thirds, would thus have to be considered as an artefact, there would still remain the one-third (ESI_{CO}) which can be split off even in inert gases. Here our experiments support Barkan's view of the bile pigment-haemoglobin nature of at least a part of this fraction. In Part 5 we shall describe the isolation of small amounts of biliverdin and biliviolin from red cells. We have not yet been able to decide whether or not the detachment of iron during the acid denaturation of haemoglobin, leads to formation of bile pigment. If the porphyrin part of the haemoglobin molecule is oxidized to substances other than bile pigment, the yield of bile pigment indicates that almost the total ESI_{CO} fraction is bile pigment-haemoglobin. Otherwise a part of the bile pigment found would also be an artefact, certainly, however, not all, since in the presence of ascorbic acid biliverdin was obtained from red cells.

A certain part of E′ must also be the blood catalase. We have recently proved [Lemberg, Norrie & Legge, 1939] that a bile pigment-haematin group occurs in catalase. A simple calculation shows, however, that the blood catalase constitutes only a small fraction of the total E′. The monomolecular reaction constant *k* of rabbit's blood catalase multiplied by the dilution is 1500 (own unpublished experiments). If the catalase factor of pure catalase ($=k$/g. catalase in 50 ml.) is 35,000, this corresponds to a catalase concentration of 86 mg. catalase in 100 ml. of blood. Catalase contains one equivalent of bile pigment-haematin iron in a molecule of mol. wt. 248,000. The ESI derived from blood catalase is, therefore, about 20 μg. per 100 ml. of blood, while the total ESI_{CO} (E′-iron) is about $\frac{15{,}000{,}000 \times 55{\cdot}8 \times 1{\cdot}5}{16{,}700 \times 100} = 750\ \mu$g. per 100 ml. Catalase forms, therefore, less than 3 % of the E. Barkan & Schales [1936] have, indeed, separated catalase from E′ by adsorption on alumina, and with the slight qualification that their separation means separation of catalase from the main bulk of E′, their experiments are in agreement with the above calculation.

SUMMARY

The findings of Barkan & Schales are confirmed that blood and haemoglobin solutions contain 5–6 % of the total iron in the form of 'labile iron' ($\mathrm{ESI_{O_2}}$) and that about two-thirds of this ($\mathrm{ESI_{O}} - \mathrm{ESI_{CO}}$) are split off only in the presence of O_2 and in the absence of reducing substances while the remaining third is split off also in inert gases and CO ($\mathrm{ESI_{CO}}$).

In contradistinction to Barkan & Schales we explain the fraction $\mathrm{ESI_{O_2}} - \mathrm{ESI_{CO}}$ as an artefact, arising by oxidation of the prosthetic group of the haemoglobin molecule by the O_2 evolved from oxyhaemoglobin by acids.

The fraction $\mathrm{ESI_{CO}}$ is at least partially attributable to a bile pigment-haemoglobin which gives rise to bile pigments when treated with acids. Only a small part of $\mathrm{ESI_{CO}}$ is due to the blood catalase.

The increase of labile iron occurring during the coupled oxidation of haemoglobin and ascorbic acid is proportional to the concentration of choleglobin formed, the latter being estimated spectrophotometrically. About 66 % of the choleglobin iron is split off by 16 hr. incubation with $N/10$ hydrochloric acid, while the remainder is found as unsplit cholehaematin in the precipitate of denatured protein. Neither CO nor reducing substances appear to inhibit the acid splitting of choleglobin.

REFERENCES

Ammundsen (1939). *Science*, **90**, 372.
Barkan (1936). *Hoppe-Seyl. Z.* **221**, 241.
—— (1937). *Klin. Wschr.* **16**, 300.
—— & Berger (1928). *Arch. exp. Path. Pharmak.* **136**, 278.
—— —— (1930). *Biochem. Z.* **224**, 53.
—— & Schales (1936). *Hoppe-Seyl. Z.* **244**, 81.
—— —— (1937). *Hoppe-Seyl. Z.* **248**, 96.
—— —— (1938). *Hoppe-Seyl. Z.* **253**, 83.
—— —— (1939). *Science*, **90**, 616.
Engel (1939). *Hoppe-Seyl. Z.* **256**, 75.
Holden (1936). *Aust. J. exp. Biol. med. Sci.* **14**, 291.
—— (1937). *Aust. J. exp. Biol. med. Sci.* **15**, 43.
Klumpp (1935). *J. clin. Invest.* **14**, 351.
Lemberg (1935). *Biochem. J.* **29**, 1322.
—— & Legge (1938). *J. roy. Soc. N.S.W.* **72**, 62.
—— —— & Lockwood (1939). *Biochem. J.* **33**, 754.
—— Norrie & Legge (1939). *Nature, Lond.*, **144**, 551.
—— & Wyndham (1937). *J. roy. Soc. N.S.W.* **70**, 343.
Lintzel & Radeff (1928). *Biochem. Z.* **203**, 212.
Rawlinson (1939). *Aust. J. exp. Biol. med. Sci.* **17**, 53.
Shorland & Wall (1936). *Biochem. J.* **30**, 1047.
Taylor & Coryell (1938). *J. Amer. chem. Soc.* **60**, 1177.

36. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN

5. STUDIES ON THE FORMATION OF BILE PIGMENTS FROM CHOLEGLOBIN AND VERDOHAEMOCHROMOGEN AND ON THEIR ISOLATION FROM ERYTHROCYTES[1]

By R. LEMBERG, W. H. LOCKWOOD and J. W. LEGGE

From the Institute of Medical Research, Royal North Shore Hospital, Sydney

(*Received 3 March 1941*)

In a preliminary paper [Lemberg, Legge & Lockwood, 1938] we have already reported the isolation of biliverdin (dehydrobilirubin) and of biliviolin from choleglobin solutions. Under remarkably mild conditions haemoglobin is thus transformed into bile pigments; these conditions are such as are found in the cells of organs forming bile pigment, except that acid was used for the removal of the iron. It will be shown in the present paper that the breakdown of choleglobin to bile pigments with the removal of iron probably occurs in the later stages of the reaction with ascorbic acid at a physiological pH.

We describe here in greater detail the bile pigments obtained by acid splitting from choleglobin and verdohaemochromogen. The hope of gaining information on the constitutional differences between cholehaem and verdohaem by the study of the splitting products was not fulfilled owing to secondary reactions by which the primary splitting products are altered and owing to the complicated nature of the mixtures of bile pigments thus formed.

Great difficulties had, therefore, to be overcome in working out quantitative methods enabling us to estimate the yield of bile pigment from the bile pigment-haematin compounds. This was, however, achieved and we can now correlate the yield of bile pigments obtained from choleglobin solutions by splitting with acids with the choleglobin concentration of these solutions.

In the above-mentioned preliminary publication we had also reported the isolation of bile pigments from erythrocytes. In the present paper we describe these experiments in greater detail.

EXPERIMENTAL

Preparation of biliverdin and biliviolins[2] from haemoglobin by coupled oxidation with ascorbic acid

In each of 12 flat-bottomed culture flasks 50 ml. of a 1·5% solution of crystalline horse oxyhaemoglobin, buffered to pH 8·5 with hydrogen phosphate, were incubated for 2 hr. with 50 mg. of ascorbic acid at 37°. The supernatant solution was poured off from the green pigment and poured into 2 vol. of glacial acetic acid. After 15 min. at room temperature this solution was poured into 2 vol. of ether (freed from peroxide over sodium) and the protein removed together with the greater part of the acetic acid by repeated washing with dilute sodium acetate solution and finally with water, avoiding shaking in the initial stages.

---

[1] This work was carried out under a grant from the National Health and Medical Research Council of Australia.

[2] According to Siedel (*Angew. Chemie*, **52**, 38 [1939]) our compounds would be 'bilipurpurins', not 'biliviolins'. Siedel restricts the name 'biliviolins' to a very similar class of substances, probably isomeric with bilirubins, one of which e.g. is formed by the oxidation of mesobilinogen by ferric chloride; for the pigments of an oxidation stage higher than the biliverdins he uses the term 'bilipurpurins'. The extraordinary similarity of the properties of 'biliviolins' and 'bilipurpurins', and the fact that the latter comprise again several pigment classes, makes it appear more practical to maintain the generic name biliviolins, for all these pigments, and to distinguish them by figures and letters, until more is known about their chemical constitution. The term also tallies better with the actual colour of the pigments.

Biliverdin was extracted from the ethereal solution by repeated extractions with a few ml. of 5 % HCl. After a few hours green flocks of biliverdin hydrochloride settled down; they were collected by filtration and washed on the filter with water. The mother liquor contained a biliviolin. The biliverdin hydrochloride was dried on the filter in a vacuum desiccator and transformed into the dimethyl ester of biliverdin in the usual way. The ester was brought once more into ether and finally recrystallized from methyl alcohol. 20 mg. of blue-green needles. M.P. (on the copper block) 216°, mixed M.P. with an ester prepared from bilirubin (M.P. 212°) was 214°.

After the extraction with 5 % HCl the ether still contained a weakly basic biliviolin and protohaemin. The former was extracted with 20 % HCl. Haemin was then extracted with dilute ammonia and transformed into the pyridine-haemochromogen by pyridine and $Na_2S_2O_4$. Only the absorption bands of protohaemochromogen could be observed. Cholehaematin must, therefore, have been transformed into the bile pigments, except that part of it which remains unsplit in the protein precipitate. The biliviolin present in the 20 % HCl extract was taken up by chloroform from the diluted solutions. The chloroform solution was washed to neutrality with water. It was then red-violet and showed a rather diffuse absorption band at 562 mμ and a second weaker band at 520 mμ.

A phenomenon characteristic of the different biliviolins is their hydrochloride formation, when a neutral chloroform phase containing the pigment is shaken with an aqueous phase containing dilute HCl. The absorption spectrum changes from that of the neutral compound to that of the hydrochloride with an absorption band in the orange part of the spectrum, while the colour changes from pink or red-violet to blue-violet or blue, the colour change being more marked in daylight than in artificial light. This change depends on the concentration of the acid in the aqueous phase and also to a remarkable degree on the temperature, a higher temperature favouring salt formation. This can only be explained by the assumption that biliviolins occur in two tautomeric forms, the more strongly basic form being unstable and favoured by higher temperature.

The biliviolin obtained from the 20 % HCl extract behaved in this chloroform-HCl test as indicated by Table 1. We shall call it biliviolin III*a*. Its hydrochloride in aqueous solution was violet, in chloroform or in methyl alcohol in daylight almost blue, in artificial light violet. Its aqueous solution showed a broad absorption band with its centre at 596 mμ, with its maximum, however, somewhat more towards the red at about 604 mμ.

Table 1. *Chloroform-HCl test of biliviolin IIIa*

Temperature	1 %	5 %	10 % HCl
0°	Neutral	Neutral	Partly acid
20°	Neutral	Neutral	Acid
60°	Neutral	Partly acid	Acid

In chloroform the band was at 622 mμ. The zinc salt in methyl alcohol was blue-green with red fluorescence and a two-banded absorption spectrum (I 646, II 581 mμ; intensities: I, II).

Table 2. *Chloroform-HCl test of biliviolin IIb*

Temperature	1%	5%	10% HCl
0°	Neutral	Partly acid	Acid
20°	Neutral	Acid	Acid
60	Acid	Acid	Acid

A second biliviolin was isolated in smaller yield from the mother liquor of biliverdin hydrochloride. It showed a different behaviour in the chloroform-HCl test (Table 2). We shall call it biliviolin II*b*. The biliviolins can either be derived directly from choleglobin, or they may be products of a secondary oxidation of biliverdin. If about 200 μg. biliverdin are dissolved in dilute ammonia and added to a 0·7% oxyhaemoglobin solution and this mixture is treated with glacial acetic acid, the biliverdin is transformed into biliviolin, which according to its properties is biliviolin II*b*. The same biliviolin had been previously obtained by oxidizing the complex zinc salt of biliverdin with two atoms of iodine per mol. and by exposing the unstable biliviolin II*a*, set free from its zinc salt, to atmospheric O_2 [Lemberg & Lockwood, 1938, and unpublished experiments]. It is therefore likely that this biliviolin arises by secondary oxidation of biliverdin during the choleglobin splitting with acids.

The choleglobin solutions contain ascorbic acid, and if ascorbic acid in the same concentration (50 mg. per 100 ml.) is added to the mixture of biliverdin and oxyhaemoglobin, there is practically no oxidation of biliverdin to biliviolin. Even in the presence of excess of ascorbic acid or of CO, biliviolin III*a* was always found to accompany biliverdin in the splitting products of choleglobin. Ascorbic acid protects biliverdin from oxidation during the action of acid on oxyhaemoglobin (cf. Table 6), but does not reduce biliviolins to biliverdin. These experiments make it likely that the biliviolin III*a* is a genuine splitting product of choleglobin. It will be shown below that verdohaemochromogen yields also biliviolin besides biliverdin in the presence of ascorbic acid, and that the primary split-product of verdohaemochromogen is apparently oxidized by ferric iron to biliviolin. This biliviolin, however, is biliviolin II*b*, the same that arises by oxidation of biliverdin, while the biliviolin III*a* from choleglobin is different.

The same split products were obtained from choleglobin if the method of splitting was varied, e.g. if an acetic acid-ethyl acetate mixture (1:2) was used, or acetic acid containing HCl or if the splitting was carried out at 100°. With acetic acid and hydrazine hydrate or with ferrous acetate in acetic acid-HCl, however, only biliverdin was isolated. In these reactions no trace of the hydroxyporphyrin described below was observed.

Split products of 'green pigment'

Cholehaemochromogen obtained from choleglobin by alkali denaturation gave the same split products as choleglobin. The 'green pigment', however, which precipitated in the further course of the coupled oxidation of haemoglobin and ascorbic acid behaved somewhat differently in spite of the identity of its absorption properties with those of the first-mentioned cholehaemochromogen. The yield of ether-soluble split products was very small, the major part of the pigment remaining in the denatured protein which precipitated in the aqueous phase on addition of sodium acetate solution. Not only the bile pigments, but also the haemin from the admixed protohaemochromogen were thus largely lost. The kind of linkage of the prosthetic groups with the protein in the 'green pigment' is evidently quite different from that in the native and in the alkali-denatured chromoproteins. This is the reason why earlier observers failed to isolate bile pigments. The green, amyl alcohol-soluble pigments, obtained by treating 'green pigment' with hot mineral acids [Edlbacher & v. Segesser, 1937] are still compounds of peptide nature, resulting from partial decomposition of the firmly attached protein groups. While their positive zinc acetate-iodine reaction (red fluorescence and absorption band at 636 mμ) proves their close relationship to biliverdin, their solubility properties reveal the presence of large hydrophilic groups. They are insoluble in ether and absolute alcohol, but easily soluble in water.

Nevertheless small amounts of ether-soluble bile pigments were obtained by splitting green pigment with 66 % acetic acid—and these were the same as those isolated from choleglobin. A slightly larger yield of biliverdin was obtained, when 'green pigment' was dissolved in 1 % ascorbic acid (no reduction occurs, the absorption band being that of acid ferric cholehaemochromogen at 610 mμ) and reduction to the ferrous haemochromogen caused by the addition of a minimum amount of $Na_2S_2O_4$ before the addition of acetic acid.

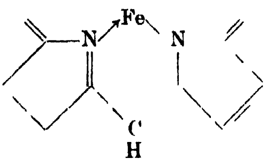
While acetic acid splitting thus yielded the same products as from choleglobin, though in much smaller yield, another split product of different nature was found, when the splitting was carried out with a ferrous acetate-acetic acid-HCl mixture or with glacial acetic acid in the presence of hydrazine hydrate. From the ether solution 0·5 % HCl extracted biliverdin, and 5 % HCl protoporphyrin and small amounts of a compound with a sharp absorption band at 627 mμ. Somewhat larger amounts of this substance were contained in the 20 % HCl extract. The yield of all split products was again small. The new compound was brought back into ether by partial neutralization of the 20 % HCl extract and separated from the last traces of protoporphyrin by repetition of the HCl fractionation. The acid solution was green without fluorescence, while the dirty yellow neutral solution in ether-acetic acid showed red fluorescence. This solution showed four absorption bands: I 684, II 592, III 544, IV 504 mμ (intensities: I, III, IV, II). The Willstätter number was about 14. The copper complex salt showed the following spectrum: I 628, II 532 mμ (intensities I, II).

The properties of the substance show that it is different from the hydroxyporphyrin isolated by Lemberg, Cortis-Jones & Norrie [1938] from its haematin which was observed as an intermediate product in the formation of verdohaemochromogen from pyridine haemochromogen with ascorbic acid. It appears, however, to bear a close resemblance to the oxymesoporphyrins of Fischer *et al.* [1927; 1930], substances of uncertain constitution arising by the action of H_2O_2 on mesoporphyrin. Probably it is the corresponding proto compound, the absorption bands lying more towards the infra-red.

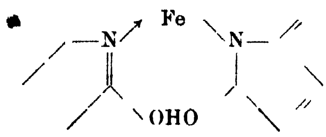
The fact that this hydroxyporphyrin cannot be obtained in the same way from choleglobin solutions, makes it unlikely that its haematin compound should be formed as intermediate product in the formation of choleglobin, or that it should be derived from cholehaematin itself. Some, though not all of Fischer's analyses indicate the presence of two oxygen atoms in addition to the four present in the two carboxylic groups of mesoporphyrin. Probably our new compound is a dihydroxyprotoporphyrin, the haematin of which is produced by a side reaction involving the oxidation of a second methene group before the ring is opened at the methene group first oxidized.

Bile pigments from verdohaemochromogen and verdohaematin

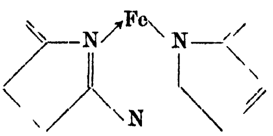
Verdohaemochromogen was shown by Lemberg [1935] to be transformed into biliverdin by hot methyl alcoholic HCl. Libowitzky & Fischer [1938] have recently claimed that the verdohaemochromogen of the copro series is not split by a mixture of equal parts of glacial acetic acid and conc. HCl. From this observation they conclude that the 'green haemin' is not a haemochromogen, but a free ferric haematin, probably not of bile pigment-haematin nature. Warburg & Negelein have, however, already shown that the typical three-banded absorption spectrum disappears when the pyridine is washed away from the chloroform solution; it also disappears when the haemochromogen is split by acetic acid or by alkali. We can show also (cf. Table 7 below) that verdohaemochromogen can be split to bile pigments with good yield under much milder conditions than those applied by Fischer. The data collected in Table 7 show that if there is any difference in the reactions of ferric verdohaematin and verdohaemochromogen with acids, the former is split more easily. Libowitzky & Fischer base their assumption also on the observation that hydrazine hydrate destroys the typical absorption spectrum. That hydrazine does not destroy 'green haemins' by reduction, is, however, evident from the fact that it is used in their preparation from haemins. Verdohaemochromogens are unstable in all strongly alkaline solutions which transform them into yellow compounds. We have recently obtained further conclusive evidence [cf. also Holden & Lemberg, 1939] for the formula of verdohaemochromogen as an *iso*biliverdin-haemochromogen with opened ring by condensing it with ammonia and transforming it thus into the haemochromogen of an azaporphyrin with nitrogen-closed ring (unpublished experiments).



Protohaem



Verdohaem



Aza-haem

Verdohaemochromogen appears to us best formulated as a resonance structure with a hydrogen linkage between oxygen atoms closing the ring.

We have now subjected verdohaematin, verdohaemochromogen and verdoglobin to the same mild splitting conditions as were used for choleglobin above. Quantitative data will be given later; here we describe the splitting products. Again under all conditions mixtures of biliverdin and biliviolin were obtained (cf. Table 6), whether an alkaline solution of ferric verdohaematin or a solution of the haemochromogen in pyridine was treated with 66 % acetic acids. On keeping at room temperature biliviolins were obtained predominantly, while on short boiling biliverdin became the main product. Mainly biliverdin was formed when the splitting was carried out in the presence of ascorbic acid. Glacial acetic acid plus hydrazine hydrate and ferrous acetate in acetic acid-HCl in the heat produced biliverdin only.

For the isolation of the biliviolins 2 vol. of glacial acetic acid were added to the freshly prepared solution of verdohaemochromogen in 2 % NaOH. After 15 min. at room temperature the solution was diluted with an equal volume of ether. Washing with water removed some unsplit verdohaematin and a little biliverdin. From the red-violet ether solution 2 % HCl extracted two biliviolins, one of which (the main product) passed from 2 % HCl into chloroform, while the second was not extracted by chloroform. In the chloroform-HCl test the first substance behaved as indicated in Table 3.

The acid aqueous solution showed an absorption band at 595 mμ, the acid chloroform solution one at 614 mμ. The neutral chloroform solution had an absorption spectrum with two bands of about equal intensities (I 552, II 512 mμ).

Table 3. *Chloroform-HCl test of biliviolin IIb from verdohaemochromogen*

Temperature	1%	5%	10% HCl
0°	Neutral	Neutral	Acid
20°	Neutral	Partly acid	Acid
60°	Partly acid	Acid	Acid

The zinc salt was blue-green with strong red fluorescence and absorption bands at 640 and 585 mμ, the former being far stronger. In spite of slight differences in the chloroform-HCl tests (cf. Tables 2 and 3) this violin is certainly identical with biliviolin II*b*.

The second biliviolin was brought into chloroform by neutralization with sodium acetate. The neutral solution had a somewhat weaker second band than that of biliviolin II*b*. 1% HCl extracted it completely from chloroform. The centre of the absorption band of its hydrochloride was at 592 mμ. This biliviolin, of which only traces are obtained, appears to be identical with that formed as a primary product by dehydrogenation of the biliverdin zinc salt with iodine (biliviolin II*a*; unpublished experiments, and Lemberg & Lockwood [1938]).

The original ether solution was further successively extracted with 5, 10 and 20% HCl. All these extracts contained violin. While the violin in the 5% HCl extract was also biliviolin II*b*, the other fractions contained a third biliviolin of weaker basicity. The absorption bands of the neutral solution in chloroform were at 554 and 514 mμ; those of the acid chloroform solution at 617 mμ. It differs from the biliviolin III*a* isolated from choleglobin by having absorption bands more towards the blue. We shall call it, therefore, biliviolin III*b*. Its behaviour in the chloroform-HCl test (Table 4) does not differ significantly from that of biliviolin III*a*.

Table 4. *Chloroform-HCl test of biliviolin IIIb from verdohaemochromogen*

Temperature	1%	5%	10% HCl
0°	Neutral	Neutral	Largely acid
20°	Neutral	Neutral	Acid
60°	Neutral	Partly acid	Acid

The comparatively strongly basic biliviolin II*b* forms the main bulk, while from choleglobin the weakly basic biliviolin III*a* was formed predominantly.

Methods for the quantitative estimation of biliverdin and biliviolin

The methods for quantitative determination of bile pigments detached from choleglobin or verdohaemochromogen solutions were based in principle on the methods of isolation described above. For an exact determination of the bile pigment yield from these substances the biliviolin cannot be neglected. In the later phases of the coupled oxidation, however, biliverdin prevails. Biliverdin was also obtained as main product from catalase [Lemberg, Norrie & Legge, 1939]. We have therefore worked out a microcolorimetric method for the determination of biliverdin alone, which is simpler and requires less material (method I). For the determination of biliviolin as well as biliverdin spectrophotometric methods were developed. In the first of these (method II) biliverdin and biliviolin are separated by the Willstätter method of fractional HCl extraction from ether and determined separately, while in the second (method III) both are extracted together and their concentrations are calculated from the extinction coefficients at the wave-lengths of the maximal extinctions of biliverdin and biliviolin at 675 and 595 mμ respectively. Method II is suitable for determination of the bile pigments from choleglobin solution, since the weakly basic biliviolin III*a* is easily separated from biliverdin, and unsuitable for the determination of the bile pigments from verdohaemochromogen, with which such a separation is difficult owing to the relatively strong basicity of biliviolin II*b*. Here and for the study of the recovery of biliverdin added to oxyhaemoglobin solutions, in which also biliverdin II*b* is formed, method III was applied.

Method I. 5 ml. of the choleglobin solution are pipetted into 10 ml. of glacial acetic acid and after 15 min. at room temperature the mixture is poured into 15 ml. of peroxide-free ether in a narrow 50 ml. separating funnel. 5 ml. of saturated sodium acetate solution are added, the tube once inverted and 10 ml. of water added. After removal of the aqueous phase the protein precipitate suspended in the ether is removed together with the main bulk of acetic acid by washing with 10 ml. of dilute sodium acetate solution. Vigorous shaking must be avoided during the initial stages. The ether solution is then washed 2–3 times with water, the last time with shaking. It is essential to have the last trace of protein removed, but on the other hand to leave enough acetic acid in the ether to prevent precipitation of haematin. The first two sodium acetate extracts are overlayered once more with a few ml. of ether and saturated ammonium sulphate solution is added, until the protein comes out in green flocks at the interface and the aqueous layer becomes colourless. The latter is removed and the ether solution is poured off from the protein, washed with dilute sodium acetate solution and added to the main ether extract.

Biliverdin is then extracted by 2 ml. of 2 % HCl, followed by two further extractions with 2 ml. of 5 % HCl each. The united extracts are made up to 10 ml. with alcohol and matched colorimetrically with a biliverdin standard containing 100 μg. biliverdin in 10 ml. 40 % alcohol with an HCl content of 2·5 %. The standard is prepared freshly each day from a stock solution of 10 mg. pure crystalline biliverdin in 100 ml. alcohol, which is kept in the dark. During 6 weeks the specific extinction coefficient (ϵ of an 0·1 % solution) of the acidified solution at the maximum of absorption ($\lambda = 675$ mμ) diminished very little (from 47·1 to 46·5).

Method II. Biliviolins cannot be estimated colorimetrically, since their solutions are not stable enough to be used as standards, and other pigments which match the colour exactly are not available. A spectrophotometric method is therefore used. Biliviolins have not yet been obtained crystalline and their specific extinction coefficients are at present unknown. The purification of the biliviolin III*a* from choleglobin in sufficient quantities promised little hope of success. We have therefore used a fresh sample of biliviolin II*b*. This was isolated from the 5 % HCl fraction obtained after splitting of verdohaemochromogen with acetic acid and extraction with ether. It was brought back into ether with sodium acetate and the acetic acid was removed by extraction with water. The evaporation residue of the ether solution was dried *in vacuo* and weighed. It was then dissolved in 2 ml. of alcohol and diluted to 10 ml. with 20 % HCl. The specific extinction coefficient at the maximum of absorption (at 595 mμ) was found to be 46·0. This value is so close to that of the maximal specific extinction coefficient of biliverdin, that it can be reasonably assumed that the sample of biliviolin was pure, and also that the maximal extinction coefficient of biliviolin III*a* from choleglobin will not be very different from that of biliviolin II*b* and biliverdin. The value 46·0 for the specific extinction coefficient of biliviolin as hydrochloride at 595 mμ and the value 46·5 for that of biliverdin as hydrochloride at 675 mμ were used to convert spectrophotometric readings into terms of concentration.

For method II 50 ml. of a choleglobin solution obtained from 0·7 % haemoglobin are required. They are worked up as described for method I, the extraction of the bile pigments being carried out, however, in a different manner and with relatively smaller volumes of HCl in order to get sufficiently strong solutions. 10–15 ml. of 5 % HCl are used for the extraction of biliverdin and the same volume of 20 % HCl for the extraction of biliviolin for samples of choleglobin solutions obtained by a short incubation of haemoglobin with ascorbic acid, while for samples of longer incubation time 25–30 ml. of the acids can be used. 5 ml. of alcohol are added and the final volume is measured. In a layer of 4–5 cm. length these bile pigment solutions give extinctions in the optimal range of the spectrophotometer. The instrument was the Hilger Visible Spectrophotometer described in Part 2.

Method III. If the extinction coefficients of both biliverdin and biliviolin are known at two wave-lengths at which they differ considerably, the content of a mixture of both substances can be established by measuring the extinctions at these wave-lengths. The wave-lengths of the absorption maxima ($\lambda = 675$ and 595 mμ) are suitable. The specific extinction coefficients of biliverdin hydrochloride were found to be 46·5 at 675 mμ and 21·4 at 595 mμ, while those of biliviolin hydrochloride were 46·0 at 595 mμ and 6·0 at 675 mμ. If ϵ_{675} and ϵ_{595} are the extinction coefficients of the solution in 1 cm. depth at the wave-lengths 675 and 595 mμ the following two equations hold:

$$\mu\text{g. biliverdin per ml.} = 2{\cdot}98\ (7{\cdot}67\ \epsilon_{675} - \epsilon_{595})$$

$$\mu\text{g. biliviolin per ml.} = 10{\cdot}64\ (2{\cdot}17\ \epsilon_{595} - \epsilon_{675}).$$

For the determination with this method the ethereal solution is extracted twice with 5 ml. each of 5 % HCl and three times with 5 ml. each of 20 % HCl. After the addition of 5 ml. of alcohol to the united extracts the volume is measured and the extinction coefficients at 675 and 595 mμ are measured.

Table 5 shows that with choleglobin methods II and III give essentially the same results, while the differences are larger when the bile pigments from verdohaematin or verdohaemochromogen are determined; here method III is preferable.

Table 5. *Determination of bile pigments from verdohaemochromogen and choleglobin*

	Method II			Method III		
	μg. verdin	μg. violin	μg. bile pigment	μg. verdin	μg. violin	μg. bile pigment
Verdohaematin	52	104	156	33	134	166
Verdohaemochromogen + ascorbic acid	167	42	209	165	106	271
Choleglobin	950	251	1201	978	203	1191

Bile pigment yield from verdohaemochromogen

Experiments on the yield of bile pigments from verdohaematin compounds were carried out in order to examine in what yield bile pigment is isolated from these bile pigment-haematin compounds of established constitution under the conditions used for the splitting of choleglobin.

Verdohaemochromogen was either dissolved directly in 66 % acetic acid (*a*) or it was first dissociated to verdohaematin by dissolving it in 1 ml. of 2 % NaOH (*b*) or dissolved in 1 ml. of 20 % pyridine (*c*) and these solutions added to 2 ml. of glacial acetic acid. Finally a solution of verdohaematin in 1 % Na_2CO_3

was coupled with native horse globin and 6 ml. of this solution of ferric verdoglobin (*d*) were added to 12 ml. of glacial acetic acid. The bile pigments were isolated as from choleglobin, the unsplit verdohaematin being removed from the ether by repeated washing with water before the extraction of the bile pigments with HCl. Method III was applied to the determination and the results are given in Table 6.

Table 6. *Bile pigment yields after acid splitting of verdohaematin compounds under various conditions*

			Total bile pigment in % of theory				Biliverdin in % of total bile pigment			
Additions to the solution before splitting	Split by	Time min.	*a* Solid verdo-haemo-chromogen	*b* Verdo-haematin in alkali	*c* Verdo-haemo-chromogen in 20% pyridine	*d* Ferric verdo-globin	*a*	*b*	*c*	*d*
—	66% acetic acid	15	13·8	25·8	6·1	14·7	16	38	35	19
		60	18·5	32·5	13·7	—	25	44	18	—
5 mg. ascorbic acid	,,	15	—	25·6	6·8	10·7	—	74	62	49
		60	25·3	34·3	12·2	—	58	89	55	—
5 mg. ascorbic acid, +CO	66% acetic acid under CO	15	—	17·0	—	12·1	—	63	—	46
		60	21·5	31·4	—	—	54	71	—	—
—	66% acetic acid + 1 ml. conc. HCl	60	19·3	—	—	—	28	—	—	—
—	66% acetic acid, boiling	2	39·5	22·6	23·7	10·4	70	55	87	58
—	5% HCl	60	—	33·0	—	—	—	65	—	—
5 mg. ascorbic acid	,,	60	—	32·6	—	—	—	73	—	—

Under the mild conditions used for the splitting of choleglobin the iron from verdohaematin compounds is not detached completely, but already to the extent of 15–35% at room temperature and up to 40% by boiling acetic acid. Neither HCl, nor a mixture of acetic and hydrochloric acids increases the yield of bile pigments substantially. The degree of splitting varies a great deal with the conditions. If there is any difference between ferric verdohaematin and ferrous verdohaemochromogen in this regard, ferric verdohaematin is split more readily. The difference is, however, not large. Thus ferrous verdohaemochromogen was observed spectroscopically to persist in the 66% acetic acid in the presence of pyridine and ascorbic acid or of pyridine, ascorbic acid and CO, while its absorption spectrum rapidly disappeared in the acetic acid in the absence of ascorbic acid; the bile pigment yield was slightly higher in the absence of ascorbic acid. In the absence of excess pyridine CO reacts in the acetic acid with verdohaem when ascorbic acid is present, yielding a characteristic CO-compound with absorption bands at 611, 541 and 502 mμ (intensities: I, II, III); when ascorbic acid is absent, CO fails to form this compound. It is, therefore, CO-verdohaem with ferrous iron; pyridine converts it into verdohaemochromogen. Again the yield of bile pigment isolated from the ferrous CO-verdohaem is either the same or slightly less than from the brown ferric verdohaematin solutions obtained by the action of acetic acid on verdohaemochromogen. Verdoglobin is split by 66% acetic acid with a yield of 10–15% and neither ascorbic acid nor CO influences the yield significantly. Under all these conditions both biliverdin and biliviolin are obtained, predominantly verdin from ferrous and violin from ferric compounds. Boiling acetic and hydrochloric acids produce less violin from ferric verdohaematin than acetic acid at room temperature. *Biliviolin IIb* contains four hydrogen equivalents less than biliverdin and one would expect, therefore, a yield of 25 % of violin, if oxidation of biliverdin by the ferric haematin iron causes the formation of the biliviolin. Actually much more biliviolin is always found, although the difference in the percentages of biliverdin in total bile pigment from a ferrous and a ferric compound is roughly 25 %. Since biliviolin is not reduced by ascorbic acid, and biliverdin is not oxidized by the small concentrations of ferric acetate set free in the solutions, it is somewhat difficult to explain these results, unless it is assumed that the unstable *iso*biliverdin set free from verdohaematin compounds is more readily oxidized than biliverdin.

Recovery of biliverdin from mixtures with oxyhaemoglobin

For the determination of the bile pigment yields from choleglobin solutions, which always contain oxyhaemoglobin, it was essential to know to what extent biliverdin can be recovered by our methods from a solution of oxyhaemoglobin. A known amount of biliverdin dissolved in dilute ammonia was added to 50 ml. of an 0·7 % solution of horse oxyhaemoglobin. This amount of oxyhaemoglobin alone gave no detectable amounts of bile pigments. In the majority of the recovery experiments 50 mg. per 100 ml. of ascorbic acid were added, this being about the ascorbic acid concentration of the majority of choleglobin solutions. It has already been mentioned above that ascorbic acid prevents the oxidation of biliverdin to biliviolin during the treatment with acid. The mixture of oxyhaemoglobin, biliverdin and usually ascorbic acid was subjected to the treatment with 66 % acetic acid as described above, and the recovered bile pigment determined by method III.

Table 7 shows that, under the conditions of the determination of the bile pigment-yield from choleglobin, between 80 and 90 % (average 84 %) of the added biliverdin can be recovered and that the percentage of recovery does not depend on the amount of biliverdin in a range between 60 and 400 μg. per 50 ml., i.e. in the range of bile pigment yields obtained from choleglobin solutions. In the absence of ascorbic acid biliverdin is oxidized to biliviolin II*b* by the action of acid on oxyhaemoglobin, but the yield of biliviolin is the same as that of biliverdin obtained in the presence of ascorbic acid.

Table 7. *Recovery of biliverdin from dilute oxyhaemoglobin solutions*

		In % of added biliverdin		
μg. biliverdin added	Ascorbic acid mg. per 100 ml.	Biliverdin	Biliviolin	Total bile pigment
62·9	50	79·0	2·2	81·2
63·5	50	79·0	0	79·0
95	50	90·7	0	90·7
189	50	95·9	0	95·9
222	50	83·0	0	83·0
222	50	68·5	0·5	69·0
262	50	90·8	0	90·8
408	50	83·0	1·9	84·9
952	50	75·0	0	75·0
62·9	—	0	85·8	85·8
95	—	2·9	86·4	89·3
				Average 84 %

Bile pigment yield from choleglobin

We found that splitting at room temperature for 15 min. in 66 % acetic acid gave optimal yields of bile pigment from solutions of choleglobin. Heating, or treatment with the acid for a longer period, or the application of stronger acids, e.g. HCl, decreased the yield. Probably more of the bile pigment-haemoglobin is split, but also more is denatured in a way which prevents the removal of the bile pigment from the protein. In Table 8 the yields of bile pigments obtained from choleglobin solutions after various times of incubation of haemoglobin with ascorbic acid are compared with the amount of choleglobin determined spectrophotometrically. A superficial examination may lead to the assumption that the bile pigment is not derived from choleglobin, since there is evidently no proportionality between the amount of choleglobin and the yield of bile pigment. While the maximum of choleglobin concentration in the solution is reached in about 30 min. at *p*H 8·5 and in about 80 min. at *p*H 7·2, the maximum of the bile pigment formation occurs only after about 7 hr. incubation; for these calculations we have left out of consideration the cholehaemochromogen of the green pigment precipitate, since little bile pigment can be isolated from green pigment, at least in the first stages of its formation. It is assumed that one mole of choleglobin of mol. wt. 16,700 yields 584 g. bile pigment, 584 being the mol. wt. of biliverdin. The mol. wt. of biliviolin is somewhat higher, but the error introduced by this in the calculations, is insignificant. The data in Table 8 show that in the first stages of the reaction only 10–15 % of the bile pigment theoretically expected from the choleglobin content of the solutions is isolated, while later up to 70 % can be recovered. Choleglobin would thus appear not to yield bile pigment by the action of acids, but to be a precursor of bile pigment or of a bile pigment-haemoglobin yielding bile pigment on treatment with acids. While such an assumption would solve the difficult problem: what is the constitutional difference between cholehaem and verdohaem? it would create new vexing problems. If the bile pigments, which are obtained from choleglobin solutions by the action of acids, are not the iron-free prosthetic group of choleglobin, what can this prosthetic group be? Apart from bile pigments and protohaemin no other compounds were isolated from choleglobin solutions treated by acids. Protohaemin is exclusively derived from accompanying haemoglobin, since it decreases exactly in proportion to the haemoglobin concentration (cf. Part 3). We have also shown in Part 4 that the greater part of the choleglobin iron is removed by incubation with *N*/10 HCl for 16 hr.

A closer examination of the data in Table 8 reveals, indeed, sufficient evidence that choleglobin is split to bile pigments with a yield of 11–12 %. Such a yield is found in the initial periods in which according to Table 3 of Part 3 (p. 344) choleglobin is the only product of the reduction. This is evident from Table 9 in which the yield of unknown by-product obtained from Table 3 of Part 3, i.e. initial haemoglobin minus (choleglobin + haemoglobin + cholehaemochromogen + protohaemochromogen), is given in the third column and compared with the percentage yield of bile pigment in the fourth column. The same yield of 11–12 % is obtained from choleglobin produced by coupled oxidation of haemoglobin with glutathione or with thiolacetic acid. With the latter again no formation of by-products was indicated by the balance sheet of the reaction. The apparent bile pigment recovery from haemoglobin is somewhat higher, but still below 20 %, where there is a slight formation of by-product (under 3 % of the initial haemoglobin), e.g. in experiments with ascorbic acid at *p*H 8·5 up to 80 min. and at *p*H 7·2 up to 30 min. incubation time. Only where the balance sheet indicates a

Table 8. *Bile pigment yields from choleglobin solutions*

Haemoglobin 0·72 %, ascorbic acid 0·1 %, 37°

*p*H	Incubation time	Choleglobin in % of initial haemoglobin	μg. biliverdin from 50 ml.	μg. total bile pigment from 50 ml.	Biliverdin in % of theory		Total bile pigment in % of theory		Method	Remarks
7·2	15 min.	8·2	58	—	5·6		—		I	
		7·6	102	—	10·6	7·3	—		I	
		5·9	42	—	5·6		—		I	
		8·2	52	112	5·1	7·8	10·9	13·5	II	
		7·6	101	170	10·5		16·1		II	
	20 min.	11·2	127	217	9·0		15·4		II	
	30 min.	18·3	123	—	5·3		—		I	
		14·2	173	—	9·7	8·4	—		I	
		10·8	138	—	10·1		—		I	
		13·5	121	327	7·4		20·0		II	
		13·5	138	352	8·5	8·6	21·6	19·3	II	
		18·3	230	377	10·0		16·4		II	
	80 min.	[20]	360	1050	14·9		43·5		II	Precipitate; [ ] = not measured, average value of choleglobin
		—	393	990	16·3	17·2	41·0	34·2	II	
		—	520	753	20·6		29·8		II	
		—	427	562	16·9		22·5		II	
	2 hr.	[20]	914	1219	36·2		48·3		II	Precipitate
	5 hr.	[20]	1230	1500	48·7		59·5		II	Precipitate
	6½ hr.	[20]	1480	1690	58·7		67·0		II	Precipitate
	16 hr.	11·5	1030	—	71		—		I	Precipitate
8·5	15 min.	14·8	95	—	5·1		—		I	
		21·7	266	—	9·7	6·5	—		I	
		9·9	58	—	4·7		—		I	
		21·7	178	388	6·5		14·2		II	
		21·0	154	239	5·8	5·6	9·0	10·1	II	
		21·0	118	188	4·5		7·1		II	

	30 min.	32·6	482	—	11·7	9·4	—		I	
		32·1	465	—	11·5		—		I	
		21·0	194	—	7·3		—		I	
		31·9	279	—	7·2		—		I	
		32	242	360	6·0	8·5	8·9	12·4	II	
		32	322	560	8·0		13·9		II	
		32	180	268	4·5		6·7		II	
		37	465	810	10·0		17·3		II	
		24	209	350	7·0		11·7		II	
		31	464	616	11·9		15·8		II	
	50 min.	28·7	336	484	9·3		13·4		II	Precipitate
	80 min.	[28]	595	—	16·9		—		I	Precipitate; if filtrate alone split: 468 $\mu g. = 13\cdot3\%$ biliverdin
		28	690	893	19·5	14·9	25·3	19·1	II	
		28	379	553	10·7		15·7		II	
		28	445	572	12·6		16·2		II	
	2 hr.	36·5	756	—	16·7	26·6	—		I	Precipitate
		21·4	990	—	36·6		—		I	
		21·0	681	857	25·8	27·2	32·5	33·6	II	
		[28]	1005	1225	28·5		34·7		II	
	3 hr.	[20]	1060	—	42·7	41·0	—		I	Precipitate
		—	990	—	39·3		—		I	
	4 hr.	[20]	1340	—	53		—		I	Precipitate; if filtrate alone split: 1170 $\mu g. = 43\%$ biliverdin
8·5	5 hr.	[20]	1505 (*a*)	—	60 (*a*)		—		I	(*a*) With precipitate. From filtrate alone: 784 $\mu g. = 31\%$. (*b*) From filtrate alone
		—	910 (*b*)	—	36 (*b*)		—		I	
	6½ hr.	[20]	1030	—	41		—		I	From filtrate alone
	7½ hr.	[20]	1060	1215	42		48·3		II	From filtrate only
		—	1190	—	47		—		I	
	8½ hr.	[20]	1010	—	40		—		I	From filtrate only
	9½ hr.	[20]	775	—	31		—		I	From filtrate only
	16 hr.	13·6	620	—	36		—		I	From filtrate only
					Thiolacetic acid					
7·2	90 min.	19·5	220	307	8·5		11·9		II	Ascorbic acid added before acidification
					Glutathione					
7·2	19 hr.	6·0	50	86	6·6		11·4		II	Ascorbic acid added before acidification

Table 9. *Correlation between by-product formation during coupled oxidation and excess bile pigment not derived from choleglobin*

*p*H	Time of incubation min.	By-product formed in % of choleglobin equivalents	Average yield of bile pigment in % of choleglobin equivalents	Average yield of excess of bile pigment over 11·5 % in equivalent % of by-product
8·5 with ascorbic acid	15	0	10·1	—
	30	0	12·4	—
	80	2·6	19·1	82
	120	8·1	33·6	73
	420	25·9	48·3	28
7·2 with ascorbic acid	15	0·4	13·5	38
	30	2·8	19·3	41
	80	9·5	34·2	48
	120	12·4	48·3	59
	420	11·4	67·0	97
7·2 with thiol-acetic acid	90	0	11·9	—

considerable formation of by-product in the later stages of the reaction, much more bile pigment is obtained. It can also be seen from Table 9 that the initial rate of bile pigment formation at *p*H 8·5 is more than double that at *p*H 7·2, exactly as in the case with the rate of choleglobin formation. From these observations we conclude that choleglobin is split by 66 % acetic acid to 11·5 %, i.e. to about the same degree as verdoglobin (cf. Table 6). This also supports the view that the two substances are closely related to each other.

We have attempted to gain information on the question to what degree choleglobin is split by the treatment with 66 % acetic acid in other ways, but have failed to obtain a definite result. The unsplit choleglobin was precipitated from the aqueous solution remaining after ether extraction of bile pigments and protohaematin by sodium acetate and ammonium sulphate. It was then filtered off, washed free from salts and redissolved in *N*/10 NaOH. The absorption maximum at 630 mμ of the CO-cholehaemochromogen was measured and its strength indicated that about 70 % of the choleglobin (in one experiment 45 %) had been split. The colour of the recovered cholehaemochromogen is, however, rather brown than green, and it is likely that it has become altered and that its absorption has been diminished in intensity. The values found, give therefore, only minimum values of recovery of unsplit choleglobin. In the case of cholehaemochromogen smaller salt concentrations are required for the precipitation of the pigment and the estimation of recovery is more likely to be correct. While incubation with *N*/10 HCl removed 66 % of the iron from green pigment 83 % were recovered unsplit after 15 min. standing at room temperature in 60 % acetic acid.

The later part of the reaction with ascorbic acid is complicated by several factors. First, 'green pigment' precipitates and while little bile pigment is obtained from 'green pigment' precipitated after 80 min. incubation, later on a larger part of the bile pigment may be derived from precipitate (cf. the values of biliverdin obtained from the solution alone (last column) with those obtained from the unfiltered suspension (column 6 of Table 8). This bile pigment from green pigment may either be derived from a cholehaemochromogen, in which the protein has been denatured in such a way that no firm linkage between prosthetic group and protein is established, or, more likely, from an iron-free bile pigment-protein compound. In Part 3 (p. 342) we have indeed reported evidence for the occurrence of some iron-free protein in the 'green pigment'. On longer incubation ascorbic acid evidently removes iron from cholehaemochromogen. The figures in the last column of Table 8 show, however, that the bile pigment recoveries from the filtrate alone also still far exceed the 11·5 % derived from choleglobin. In the last column of Table 9 the bile pigment derived from choleglobin, assuming a yield of 11·5 %, is subtracted from bile pigment found, and this excess is given in equivalent percentage of the unknown by-product. This percentage cannot be established with great accuracy, but the figures leave no doubt that the by-product yields a much larger amount of bile pigment than choleglobin or verdoglobin. It is therefore likely that prolonged incubation of haemoglobin with ascorbic acid sets free bile pigment from choleglobin, and that this occurs now in the solution still bound to protein, but no longer to iron. This excess bile pigment not derived from choleglobin consists almost entirely of biliverdin. Since biliverdin is reduced by $Na_2S_2O_4$ to a yellow compound with no absorption in the red and green parts of the spectrum, the spectrophotometric determination of the cholehaem and protohaem compounds is not affected by its presence, and in the balances of Table 3, Part 3 the by-product appears simply as deficit.

Additions of further ascorbic acid before the acid splitting of choleglobin or of CO do not alter the total yield of bile pigment; they increase the yield of biliverdin slightly and decrease the yield of biliviolin correspondingly.

Isolation of bile pigments from red cells

The bile pigment yield from red cells is small and for its estimation rather strong solutions of haemoglobin must be employed. It is to be expected that the recovery of bile pigment from such solutions will be less complete than from dilute haemoglobin solutions. On the other hand the yield of bile pigment obtained from the blood or from strong solutions of haemoglobin can no longer be neglected in the estimation of the recovery of added bile pigment. We therefore measured the amount of bile pigment that can be isolated from red cells in one sample and determined the degree of recovery of added biliverdin in a second sample of the same red cell solution. In order to avoid oxidation of the biliverdin, ascorbic acid (50 mg. per 100 ml.) was added immediately before acidification with acetic acid.

20 ml. of fresh sheep cells, twice washed with saline were diluted to 100 ml. with water and haemolysed. 50 ml. of this solution were treated with ascorbic acid and acetic acid, while to the second lot of 50 ml. a solution of 222 μg. biliverdin in 1 ml. dilute ammonia was added before they were treated in the same way. Both samples were worked up in the manner described with a few modifications. With the high protein concentrations great care had to be taken to avoid the formation of emulsions. The greater part of the protein was precipitated by the addition of somewhat more ether than was used for the recovery of bile pigment from dilute solution, and filtered off before the addition of sodium acetate solution and water. It was impossible to avoid precipitation of some haemin from the ether before complete removal of the protein; this was filtered off from the ethereal solution, or if there was not so much as to disturb the separation of the layers, from the acid extracts, which had to be filtered in any case. Both samples were worked up in precisely the same manner. 62 μg. bile pigment (42 μg. biliverdin and 16 μg. biliviolin) were isolated from the first sample and 162 μg. biliverdin from the second. From the added 222 μg. thus only $162-46=116$ μg. $=52$ % were recovered.

If it is assumed that the same percentage of the bile pigment set free by splitting the bile pigment-haemoglobin of the red cells is recovered as from added biliverdin, and that the bile pigment-haemoglobin of the red cells is split to the same degree as choleglobin or verdoglobin, the amount of bile pigment found has to be multiplied by the factor $\frac{100}{52}\cdot\frac{100}{11\cdot 5}\cdot\frac{16{,}700}{584}=480$ in order to obtain the amount of bile pigment-haemoglobin, from which it was derived. 62 μg. bile pigment thus correspond to 29·8 mg. bile pigment-haemoglobin. Since the 10 ml. of laked cells contained 3270 mg. haemoglobin, only 0·9 % of the red cell haemoglobin was bile pigment-haemoglobin.

In a second sample 8·75 ml. of sheep erythrocytes containing 2·8 g. haemoglobin yielded 66·2 μg. bile pigment (45·7 μg. biliverdin and 20·5 μg. biliviolin) without the addition of ascorbic acid, while with ascorbic acid only 46 μg. (23 μg. biliviolin and 23 μg. biliverdin) were obtained. These values correspond to bile pigment contents of 1·1 and 0·8 %.

Horse erythrocytes were not studied quantitatively, but qualitatively they gave the same results. Similar yields to those from sheep red cells were obtained from solutions of crystalline horse oxyhaemoglobin. 50 ml. of a 5·7 % solution gave 76 μg. bile pigment (39 μg. verdin and 37 μg. violin), corresponding to a bile pigment-haemoglobin content of 1·3 %, when CO-haemoglobin was split in the presence of 50 mg. ascorbic acid, while the splitting of the oxyhaemoglobin without ascorbic acid gave here a smaller yield of 35 μg. (of which 30 μg. were violin) corresponding to 0·6 % bile pigment-haemoglobin.

These estimations are not very accurate, but while proving the presence of some bile pigment-haemoglobin in red cells, they make it unlikely that bile pigment-haemoglobin constitutes more than a part of the 'labile iron' of the blood. Since splitting in the presence of ascorbic acid and of CO decreases the yield of bile pigment little, if at all, the latter cannot be derived to a large extent from oxidative processes occurring during the acidification of oxyhaemoglobin solutions, but must be derived, at least partly, from bile pigment-haemoglobin preformed in the red cells. The biliviolin obtained appeared to be of the weakly basic type III, but not sufficient material was available to identify it with the biliviolin III*a* obtained from choleglobin.

SUMMARY

On splitting with acetic acid choleglobin yields biliverdin and a weakly basic biliviolin (biliviolin III*a*). Cholehaemochromogen ('green pigment') gives small amounts of the same products, but the greater part of the prosthetic group has become firmly attached to the denatured protein. On splitting with ferrous acetate-acetic acid-hydrochloric acid green pigment yields small amounts of a hydroxyporphyrin. Verdohaemochromogen is split by acids to biliverdin and a biliviolin of medium basicity (biliviolin II*b*).

Quantitative methods are worked out allowing the isolation of bile pigments from solutions that contain oxyhaemoglobin, and for their spectrophotometric and colorimetric determination. An average recovery of 85 % is obtained. Biliverdin is oxidized during the action of acids on oxyhaemoglobin to biliviolin II*b*, and this oxidation is prevented by ascorbic acid.

Verdohaematin is split to bile pigments by 66 % acetic acid to an extent of 25–35 %, verdoglobin to 10–15 %. The bile pigment yield from choleglobin is 11–12 % in the initial phases of the coupled oxidation of haemoglobin and ascorbic acid, in which no by-product is formed. Later considerably more bile pigment is obtained. This bile pigment, not derived from choleglobin, is biliverdin and the by-product is probably an iron-free biliverdin-protein compound. Prolonged action of ascorbic acid on choleglobin is thus able to remove the iron held in complex combination in choleglobin and to set free biliverdin at a physiological pH.

Small amounts of bile pigments (biliverdin and biliviolin) were obtained by treatment with acetic acid from laked red cells of sheep and horse as well as from crystalline horse oxyhaemoglobin. Provided that the bile pigment haemoglobin, from which these bile pigments are derived, is split to the same degree as choleglobin, and that the percentage of recovery of these bile pigments is the same as that of added biliverdin (52 %), only about 1 % of the red cell iron is bile pigment-haemoglobin iron.

REFERENCES

Edlbacher & v. Segesser (1937). *Naturwissenschaften*, **25**, 461, 557, 667.
Fischer, Gebhardt & Rothhaas (1930). *Liebigs Ann.* **482**, 1.
—— Halbig & Walach (1927). *Liebigs Ann.* **452**, 268.
Holden & Lemberg (1939). *Aust. J. exp. Biol. med. Sci.* **17**, 133.
Lemberg (1935). *Biochem. J.* **29**, 1322.
—— Cortis-Jones & Norrie (1938). *Biochem. J.* **32**, 171.
—— Legge & Lockwood (1938). *Nature, Lond.*, **142**, 148.
—— & Lockwood (1938). *J. roy. Soc. N.S.W.* **72**, 69.
—— Norrie & Legge (1939). *Nature, Lond.*, **144**, 551.
Libowitzky & Fischer (1938). *Hoppe-Seyl. Z.* **255**, 209.

37. PYRUVIC DEHYDROGENASE OF *BACTERIUM COLI*

By JACK LESLIE STILL

From the Biochemical Laboratory, Cambridge

(*Received 31 January 1941*)

Bacterium coli oxidizes pyruvate to CO_2 and water [Cook & Stephenson, 1928]; and in the presence of dinitrophenol or NaN_3 the oxidation is complete [Clifton & Logan, 1939]. Anaerobically, the main products of the breakdown of pyruvate are acetic, lactic, formic and succinic acids, ethyl alcohol, H_2 and CO_2 [Tikka, 1935]. The theories concerning the oxidation have been summarized by Mazza & Cimmine [1934]. More recent work appeared in two papers by Krebs [1937, 1, 2].

Cell-free extracts of *Bact. coli* prepared by crushing the cells in the Booth-Green mill were found to contain an enzyme system capable of oxidizing pyruvate. This paper reports the study of some of the properties of such extracts.

I. *Methods*

Pyruvic acid was estimated by the bisulphite fixation method of Clift & Cook [1932]. The cocarboxylase was prepared by Dr H. Weil-Malherbe, to whom the author is grateful.

The activity of the enzyme system was followed by measuring the O_2 uptake in Warburg manometers at 37°. Anaerobically, methylene blue acts as hydrogen acceptor for the reaction.

II. *Preparation of the extracts*

Bacterium coli-commune Escherich (National Collection of Type Cultures No. 86) was grown on agar with tryptic digest of casein, lactate and yeast water. Yeast water was added to ensure an adequate supply of substances required for synthesis of cocarboxylase [Silverman & Werkman, 1939]. After 24–30 hr. growth the cells were washed off, centrifuged and crushed for 2 hr. in the wet-crushing mill [Booth & Green, 1938]. The crushed cells were diluted with an equal volume of distilled water and centrifuged at 12,000 r.p.m. for 40 min. The clear yellow supernatant fluid was dialysed for 14 hr. against running water.

III. *Carboxylase*

The presence of carboxylase in the fresh undialysed extracts could not be shown under aerobic conditions. Similar extracts of crushed yeast cells showed high carboxylase activity under the same conditions. This confirms the conclusion of Cook [1930] that pyruvate is not decarboxylated by *Bact. coli* in the same way as by yeast.

IV. *Components of the system*

Lipmann [1939, 3 for summary] has obtained from *Lactobacillus delbrückii* (an anaerobe which does not oxidize acetate) phosphate extracts which oxidize pyruvate to acetate and CO_2. The oxidizing system comprises at least the following: (1) a protein or proteins, (2) inorganic phosphate, (3) cocarboxylase, (4) a metal, Mg, Mn or Co and (5) flavin adenine dinucleotide.

The dialysed extracts of *Bact. coli* resemble Lipmann's preparations in several respects, as indicated in Table 1.

Table 1. *Components of the oxidizing system*

	Additions to manometers (ml.)				
Extract	1·0	1·0	1·0	1·0	1·0
M/30 phosphate *p*H 6·17	—	0·9	0·9	0·9	0·9
Cocarboxylase 100 μg./ml.	0·4	0·4	0·4	—	0·4
Manganese 100 μg./ml.	0·3	0·3	0·3	0·3	—
M pyruvate	0·2	0·2	—	0·2	0·2
Water	1·3	0·4	0·6	0·8	0·7
O_2 uptake, μl./60 min.	49·5	235·0	17·5	32·0	143·0

Phosphate, cocarboxylase and a metal are apparently involved in the oxidation of pyruvate by the dialysed extract. The degree of dependence upon added phosphate varied greatly in different preparations. Flavin adenine dinucleotide caused a small increase in the rate of oxidation. It was possible to treat the extract with ammonium sulphate up to 62 % of saturation and retain the active system in the precipitate, most of which dissolved in glass-distilled water to give an orange-yellow solution. Such treatment removed substrate, cocarboxylase and much of the metal component (Table 2).

Table 2. *Components of the precipitated system after dialysis*

	Additions to manometers (ml.)				
Enzyme	1·0	1·0	1·0	1·0	1·0
M/15 phosphate *p*H 6·17	—	1·5	1·5	1·5	1·5
Cocarboxylase 100 μg./ml.	0·2	0·2	0·2	-	0·2
Manganese 100 μg./ml.	0·3	0·3	0·3	0·3	—
M pyruvate	0·2	0·2	—	0·2	0·2
Water	1·5	—	0·2	0·2	0·2
O_2 uptake, μl./45 min.	141·0	138·5	0	3·5	39·0

(1) *Protein.* By fractional precipitation of the extract it was found that the precipitate obtained by the addition of ammonium sulphate up to 40 % saturation contained very little activity. This fraction was not completely soluble when re-suspended in water and dialysed. It contained a very active lactic dehydrogenase. The precipitate obtained between 40 and 62 % saturation with ammonium sulphate contained most of the pyruvic enzyme. This fraction re-dissolved in glass-distilled water to give a greenish yellow solution which became slightly opalescent after dialysis. This fraction also contained some of the lactic dehydrogenase. This separation was not always complete with every preparation. However, it was observed that while the 40–62 % fraction was always more active, its activity could be greatly increased by the addition of some of the 0–40 % fraction. The combined activity was considerably more than the activity of the two fractions separately (Table 3). The factor present in the 0–40 % fraction responsible for this phenomenon was non-dialysable and heat-labile. In one instance both fractions were inactive before mixing.

As it seems likely that this system is similar to that of *Lactobac. delbrückii*, the effect may be due to the partial separation of two enzymes. The reaction, as Lipmann pictures it, involves two coenzymes or prosthetic groups which have so far been considered as owing their biological activity to their union with specific proteins. In the case of the pyruvic dehydrogenase, the question arises whether cocarboxylase and flavin adenine dinucleotide have a specific protein

Table 3. *Activities of the fractions*

	Additions to manometers (ml.)						
Fraction 0–40 %	0·5	0	0·5	0·5	0·5	0·5	0·5
Fraction 40–62 %	—	1·0	1·0	1·0	1·0	1·0	1·0
M/20 phosphate *p*H 6·17	1·0	1·0	1·0	1·0	1·0	—	1·0
M pyruvate	0·2	0·2	0·2	0·2	0·2	0·2	—
Cocarboxylase 100 μg./ml.	0·2	0·2	0·2	0·2	—	0·2	0·2
Manganese 100 μg./ml.	0·3	0·3	0·3	—	0·3	0·3	0·3
Water	1·0	0·5	—	0·3	0·2	1·0	0·2
O_2 uptake, μl./40 min.	14·5	172·0	353·0	52·5	9·0	370·0	8·0

each, or one protein for the two groups. If there are two, the accelerating effect obtained after mixing the two fractions may be due to the fact that one of the proteins is almost completely absent from one of the fractions.

(2) *Inorganic phosphate.* The necessity for this component of the system could be demonstrated for certain only with enzyme extract after dialysis and before precipitation with ammonium sulphate (Table 1). Ammonium sulphate of B.D.H. A.R. standard was used throughout. Attempts to obtain preparations showing no oxidation unless phosphate was added were unsuccessful. Extensive dialysis inactivates the enzyme; so also does heating to 55–60°, which was a useful method for removing inactive protein from bacterial extracts containing soluble dehydrogenases [Still, 1940, 1].

Arsenate was found to replace phosphate in facilitating the oxidation of pyruvate by the dialysed extract (Table 4). This recalls the fact that arsenate will replace the phosphate required for the oxidation of 3-phosphoglyceraldehyde to 3-phosphoglyceric acid [Still, 1940, 2].

Table 4. *Effect of arsenate and phosphate*

Manometers contained 1·0 ml. dialysed extract, 20 μg. cocarboxylase, 30 μg. Mn and 0·2 ml. *M* pyruvate. Total volume 3·2 ml.

	O_2 uptake μl./60 min.
No phosphate	26
0·4 ml. *M*/5 phosphate	121
0·4 ml. *M*/5 arsenate	107

Lipmann has shown that when pyruvate is oxidized by dried cells of *Lactobac. delbrückii* in the presence of adenylic acid, there is a simultaneous phosphorylation of the adenylic acid. Depending largely upon the amount of adenylpyrophosphatase present in the cells and its degree of inhibition by NaF, the accumulation of adenylpyrophosphate could be demonstrated in the reaction mixture [Lipmann, 1939, 1].

Using the dialysed extract of crushed cells of *Bact. coli* no conclusive evidence could be adduced for the accumulation of adenylpyrophosphate, even in presence of *M*/32 NaF (Table 5). It was found in these experiments that the incubation of the extract with adenylic acid caused a considerable increase in inorganic phosphate whether or not pyruvate had been oxidized at the same time. In the absence of adenylic acid, incubation of the extract with or without pyruvate produced only a small increase in inorganic phosphate. *Bact. coli* rapidly dephosphorylates and then deaminates adenylic acid [Lutwak-Mann, 1936]. Both NaF and adenylic acid caused some inhibition of the rate of oxidation of pyruvate.

After precipitation with ammonium sulphate, the O_2 uptake of the fraction precipitated between 40 and 62 % saturation was considerably increased by the

Table 5. *Oxidation and phosphorylation by dialysed extract*

		Additions to manometers (ml.)			
Extract		1·0	1·0	1·0	1·0
M/5 phosphate pH 6·17		0·3	0·3	0·3	0·3
Cocarboxylase 100 μg./ml.		0·4	0·4	0·4	0·4
Manganese 100 μg./ml.		0·3	0·3	0·3	0·3
M pyruvate		—	0·4	0·4	—
M NaF		0·1	0·1	0·1	0·1
0·25M adenylic acid		—	—	0·3	0·3
Water		0·7	0·3	—	0·4
O$_2$ uptake, μl./90 min.		15·0	563·0	426·0	27·5
	Initial				
Inorganic P, mg.	1·81	2·02	1·90	3·02	3·07
Hydrolysable P, mg.	0·05	0·01	0	0	0·06

Phosphate removed by 7 minutes' boiling with N HCl was considered as having been derived from adenylpyrophosphate.

addition of inorganic phosphate (Table 6). Using this fraction it was possible to show the formation of adenylpyrophosphate during the oxidation of pyruvate (Table 7). Presumably the adenylpyrophosphatase was largely removed or inactivated during fractionation with ammonium sulphate. The oxidation of pyruvate by this fraction was not inhibited by NaF (Table 6).

Table 6. *Components of the active fraction*

	Additions to manometers (ml.)					
Fraction 40–62 %	1·0	1·0	1·0	1·0	1·0	1·0
M/5 phosphate pH 6·17	0·5	—	0·5	0·5	0·5	0·5
Cocarboxylase 100 μg./ml.	0·4	0·4	0·4	—	0·4	0·4
Manganese 100 μg./ml.	0·3	0·3	0·3	0·3	—	0·3
M NaF	0·1	0·1	0·1	0·1	0·1	—
0·8M pyruvate	0·5	0·5	—	0·5	0·5	0·5
Water	0·4	0·9	0·9	0·8	0·7	0·5
O$_2$ uptake, μl./60 min.	145·0	37·0	0	11·0	16·0	156·0

Table 7. *Oxidation and phosphorylation by active fraction*

		Additions to manometers (ml.)			
Fraction 40–62 %		1·0	1·0	1·0	1·0
M/5 phosphate pH 6·17		0·3	0·3	0·3	—
Cocarboxylase 100 μg./ml.		0·4	0·4	0·4	0·4
Manganese 100 μg./ml.		0·3	0·3	0·3	0·3
M NaF		0·1	0·1	0·1	0·1
0·8M pyruvate		0·5	0·5	—	0·5
0·25M adenylic acid		—	0·6	0·6	—
Water		0·6	—	0·5	0·9
O$_2$ uptake, μl./160 min.		340·0	489·0	0	87·5
	Initial				
Inorganic P, mg.	2·08	2·10	2·59	2·75	0·34
Hydrolysable P, mg.	0	0	0·14	0	0

(3) *Cocarboxylase.* Table 8 shows the response of the dialysed extract to the addition of cocarboxylase. No special precautions were taken to incubate the enzyme and coenzyme together before the reaction for any time longer than the equilibrium period of the manometers.

Tables 1, 2 and 3 show that most of the cocarboxylase can be removed by dialysis and practically all of it by precipitation of the enzyme with ammonium

Table 8. *Effect of cocarboxylase on oxidation of pyruvate*

Manometers contained 1·0 ml. dialysed extract, 1·6 ml. $M/100$ phosphate *p*H 6·17, and 0·2 ml. M pyruvate. Total volume 3·2 ml.

Cocarboxylase μg./3·2 ml.	μl. O_2/41 min.
—	10
2·5	86
5·0	143
7·5	171
10·0	181
15·0	184
20·0	191

sulphate and subsequent dialysis. The fact that reactivation of the enzyme by recombination with cocarboxylase takes place after dialysis indicates a point of difference between this enzyme and carboxylase. Green *et al.* [1940] have shown that once cocarboxylase has been dialysed from carboxylase, subsequent addition of cocarboxylase does not restore the lost activity.

(4) *Metal component.* Addition of Mn, Mg or Co to the dialysed extract increased the rate of oxidation of pyruvate in the presence of cocarboxylase. The increase was more marked if the enzyme had been precipitated with ammonium sulphate and dialysed, or incubated for some time and dialysed again (Table 9). Precipitation with ammonium sulphate at an acid reaction was found to be necessary in order to remove the metal component from the pyruvic dehydrogenase system of *Lactobac. delbrückii* [Lipmann, 1939, 3]. Tables 1, 2, 3 and 6 show the partial removal of this component when the extracts are precipitated.

Table 9. *Effect of metals*

The dialysed extract was incubated at 37° for 45 min., diluted once and dialysed once again for 2 hr. against distilled water. Manometers contained 1·0 ml. enzyme, 0·5 ml. $M/5$ phosphate, 0·2 ml. M pyruvate and 20 μg. cocarboxylase. Total volume 3·2 ml.

	μl. O_2/30 min.
No addition	0
Manganese, 30 μg.	64·5
Magnesium, 30 μg.	60·0
Cobalt, 30 μg.	15·0

(5) *Flavin component.* Addition of flavin adenine dinucleotide (F-A-D) to the dialysed extract increased the rate of oxidation of pyruvate by about 30 %. In view of the participation of F-A-D in the oxidation of pyruvate by extracts of *Lactobac. delbrückii* [Lipmann, 1939, 2], attempts were made to split reversibly the enzyme from *Bact. coli.* The dialysed extract was half saturated with ammonium sulphate at *p*H 3·0 and at 0°. Little of the resulting precipitate was soluble when re-suspended in water. The suspension, when tested under the standard conditions, did not oxidize pyruvate in the presence or absence of F-A-D. While the above procedure was followed by Lipmann [1939, 2] for phosphate extracts of *Lactobac. delbrückii,* less drastic treatment would seem to be more suitable for the extracts of *Bact. coli.*

V. *Reaction with molecular oxygen*

p*H*. The optimum *p*H for the oxidation of pyruvate by the dialysed extract is about 6·2. Fig. 1 shows the relation between *p*H and the rate of O_2 uptake in $M/8·9$ phosphate. Actually the O_2 uptake is quite appreciable in the absence of added buffer. Substitution of citrate or acetate buffer markedly inhibits the reaction. This effect of citrate may be due to its combination with the metal component.

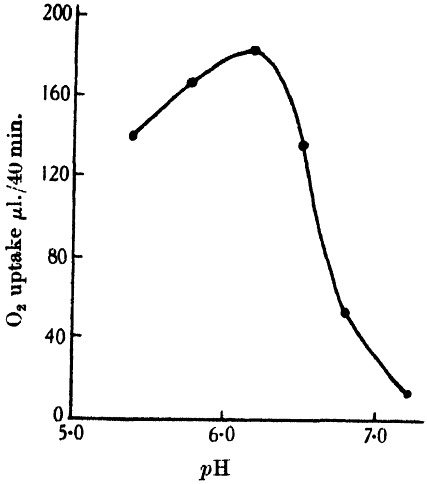


Fig. 1. Effect of *p*H on oxidation of pyruvate. Warburg manometers contained 1·0 ml. dialysed extract, 0·2 ml. cocarboxylase 100 µg./ml., 0·2 ml. *M* pyruvate, 1·8 ml. *M*/5 phosphate buffer. Total volume 3·2 ml.

Substrate. A considerable concentration of pyruvate is required to saturate the enzyme (Table 10), and it was found that pyruvate remained in the reaction mixture even after 3–4 hr. incubation.

Table 10. *Effect of concentration of pyruvate*

Manometers contained 1·0 ml. dialysed extract, 1·6 ml. *M*/15 phosphate *p*H 6·17, 20 µg. cocarboxylase. Total volume 3·2 ml.

Concentration of pyruvate m-Mol./l.	µl. O_2/25 min.
—	0
3·0	24·5
6·0	37·5
12·0	62·5
25·0	85·0
62·0	122·5
125·0	119·5

Phenyl pyruvate, at a concentration of *M*/80, was not oxidized under the same conditions.

Oxygen equivalent. The O_2 uptake per molecule of pyruvate oxidized is the equivalent of one atom of oxygen per molecule of pyruvate (Fig. 2). This result corresponds to the reaction (1):

$$CH_3.CO.COOH + \tfrac{1}{2}O_2 = CH_3.COOH + CO_2. \qquad (1)$$

Fig. 3 shows the O_2 uptake and CO_2 output during the oxidation of pyruvate, measured simultaneously by the indirect method of Warburg. As the residual pyruvate was not determined in this instance, the percentage of theoretical O_2 uptake and CO_2 output was greater than is indicated in the figure. It will be seen that the average R.Q. over 2 hr. was 0·45 against 0·50 which would be required by (1). Fig. 3 also shows the anaerobic CO_2 output over the same period. This did not reach one-quarter of the aerobic CO_2 output, which fact would seem to eliminate the possibility that pyruvate is not oxidized directly but is first dismuted according to (2):

$$2CH_3.CO.COOH = CH_3.CHOH.COOH + CH_3.COOH + CO_2, \qquad (2)$$

and that the subsequent O_2 uptake is due to direct oxidation of lactate.

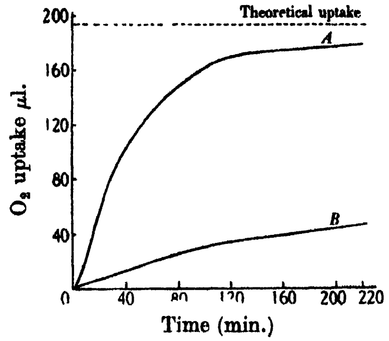


Fig. 2.

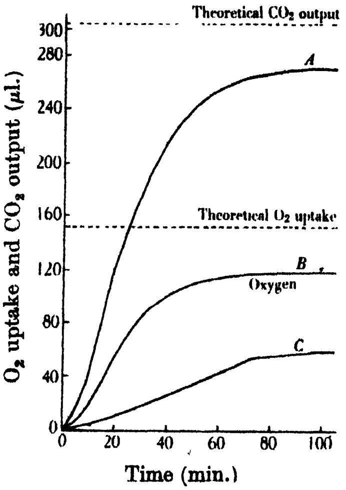


Fig. 3.

Fig. 2. Oxidation of pyruvate (*A*) and acetaldehyde (*B*). *A*, Warburg manometers contained 1·5 ml. enzyme (40–62 % fraction), 0·7 ml. *M*/5 phosphate buffer *p*H 6·17, 0·4 ml. cocarboxylase 100 µg./ml., 0·3 ml. Mn 100 µg./ml., 0·3 ml. 0·07 *M* pyruvate. Total volume 3·2 ml. Blank without substrate subtracted. *B*, as above but containing 0·3 ml. *M* acetaldehyde in place of pyruvate.

Fig. 3. Oxidation and dismutation (*C*) of pyruvate. Warburg manometers contained 1·7 ml. enzyme (40–62 % fraction), 0·6 ml. *M*/5 phosphate buffer *p*H 6·17, 0·4 ml. cocarboxylase 100 µg./ml., 0·3 ml. Mn 100 µg./ml., 0·2 ml. 0·07 *M* pyruvate. Total volume 3·2 ml. *A*, CO_2 output. *B*, O_2 uptake. *C*, CO_2 output. In *C*, the gas space contained N_2 instead of air.

Krebs [1937, 2], using intact cells of *Bact. coli*, found that under neutral or slightly acid conditions pyruvate was broken down mainly according to (3):

$$CH_3.CO.COOH + H_2O = CH_3.COOH + HCOOH. \qquad (3)$$

This reaction had been suggested previously by Tikka [1935] to account for the acetic and formic acids produced in *Bact. coli* fermentation. Under more acid conditions and in the presence of 1 atmosphere of CO_2, the process approximated more closely to (2), which Krebs found applicable to the breakdown of pyruvate by cocci and animal tissues. Barron & Lyman [1939] discuss these views in so far as they relate to certain cocci.

VI. *Product of oxidation*

Acetic acid was detected by its reaction with lanthanum nitrate [Krüger & Tschirch, 1930]. The absence of propionic acid was considered a reasonable assumption under the conditions of the experiments.

Acetic acid was estimated by the method of Friedemann [1938] owing to the presence of residual pyruvate which is to some extent volatile in steam. The following were incubated for 4 hr. in a Krebs vessel, O_2 being bubbled through the mixture: 12 ml. enzyme solution (40–62 % fraction of the extract), 600 μg. cocarboxylase, 400 μg. Mn, 8 ml. $M/5$ phosphate pH 6·17 and 59·8 mg. pyruvic acid brought to pH 6·17, the total volume being 22 ml. After the incubation the mixture was made up to 25 ml. and the residual pyruvate and the acetic acid formed were estimated. Two distillations were made; one, a steam distillation in the presence of tungstate and $MgSO_4$ and then a redistillation in the presence of tungstate, $MgSO_4$ and $HgSO_4$. This procedure was followed in order to exclude lactic, pyruvic or formic acid from the final titration. A satisfactory recovery of the acid was obtained; thus, 24·94 mg. pyruvic acid disappeared and 17·38 mg. acetic acid were recovered. The amount calculated from reaction (1) would be 17·0 mg.

If similar experiments were carried out and the reaction mixture was deproteinized by heating with dilute H_2SO_4, residual pyruvic acid could be demonstrated as the 2:4-dinitrophenylhydrazone. In addition the mixture contained a substance giving a 2:4-dinitrophenylhydrazine derivative insoluble in $NaHCO_3$, and presumably not acidic. This question has not been further investigated, but the matter recalls the observations of Quastel & Wooldridge [1929] on the products of fermentation of pyruvate by *Bact. coli*.

VII. *Oxidation of acetaldehyde*

The extract oxidized acetaldehyde under the same conditions as it oxidized pyruvate; i.e. without the addition of coenzyme I or methylene blue. However, as will be seen in Fig. 2, the rate of oxidation was small. This does not support the view that acetaldehyde as such is an intermediate in the oxidation of pyruvate to acetate. During the oxidation of pyruvate it was not possible to detect the presence of acetaldehyde using Rimini's reaction [Bernhauer, 1936]. Acetate is not oxidized by the preparation. The ability to oxidize acetaldehyde disappears after heating to 55° [Still, 1940, 1].

VIII. *Anaerobic breakdown of pyruvate*

Anaerobically, the intact cells liberated CO_2 from pyruvate, oxaloacetate and acetoacetate, but not from mesoxalate or α-ketoglutarate. These experiments were carried out in N_2 and phosphate buffer at pH 6·0. In $NaHCO_3$-CO_2 buffer pyruvate was also decomposed, the optimum pH being about 7·2. The anaerobic reaction has not been sufficiently studied to justify any statement as to the relative importance of reactions (2) and (3) under these conditions.

Fresh undialysed extract of the crushed cells decomposed pyruvate in $NaHCO_3$-CO_2 buffer at pH 7·2 at a rate which was increased by the addition of cocarboxylase. The same extract also liberated CO_2 from pyruvate in N_2 and phosphate at pH 6·0, and the latter reaction could be increased by addition of cocarboxylase (Table 11). Adenylic acid and coenzyme I were without effect.

Table 11. *Effect of cocarboxylase on anaerobic decomposition*

Manometers contained 1·0 ml. undialysed extract, 0·2 ml. M pyruvate, 1·5 ml. $M/15$ phosphate pH 6·0. Total volume 3·2 ml. N_2 in the gas space.

	μl. CO_2/60 min.
Without pyruvate	4·5
With pyruvate	60·0
With pyruvate + 20 μg. cocarboxylase	89·0

This anaerobic reaction under acid conditions was not marked, and after dialysis, or treatment with ammonium sulphate and subsequent dialysis, it was considerably diminished. Activity was not restored by the addition of riboflavin, which has been found to be essential for the dismutation of pyruvate by air-dried cells or phosphate extracts of *Lactobac. delbrückii* anaerobically under acid conditions [Lipmann, 1939, 3]. This result suggests that the reaction occurring in the extract of *Bact. coli* is probably not dismutation by reaction (2). However, the absence of a dismutation mechanism in cells grown and maintained aerobically, may be quite normal.

IX. *Oxidation of lactate*

In view of the probability that dismutation of pyruvate is brought about by the system, pyruvic dehydrogenase—carrier—lactic dehydrogenase, the occurrence of a very active lactic dehydrogenase in the cell-free extracts of *Bact. coli* is significant. The enzyme used in the experiments of Table 12 was the fraction of the extract precipitated by 40 % saturation with ammonium sulphate and then dialysed. The results in Table 12 agree with those obtained by Stephenson [1928] working with a soluble lactic dehydrogenase prepared from autolysed cells of *Bact. coli*. The enzyme does not require coenzyme I, is not inhibited by the product of the oxidation and requires a carrier in order to react with molecular O_2. This enzyme seems to be identical with the lactic dehydrogenase of yeast recently obtained in a very pure state by Dixon & Zerfas [1939].

Table 12. *Oxidation of lactate*

	Additions to manometers (ml.)				
Enzyme, 0–40 % fraction	0·5	0·5	0·5	0·5	0·5
M/10 phosphate *p*H 7·3	1·5	1·5	1·5	1·5	1·5
2 *M* lactate	0·1	—	0·1	0·1	0·1
Coenzyme I (27 % pure) 10 mg./ml.	0·1	0·1	—	0·1	0·1
Methylene blue 0·5 %	0·5	0·5	0·5	—	0·5
2 *M* NaCN	0·2	0·2	0·2	0·2	—
Water	0·3	0·4	0·4	0·8	0·5
O_2 uptake, μl./21 min.	222·0	25·0	238·0	19·0	250·0

Discussion

From the results of this investigation it would seem probable that *Bact. coli* contains a pyruvic oxidizing system very similar to the pyruvic dehydrogenase of *Lactobac. delbrückii*. Until the enzyme is reversibly split or other evidence for the participation of flavin adenine dinucleotide in the oxidation is obtained, complete identity remains in doubt. Under anaerobic conditions, the 'hydroclastic' reaction (3) is probably much more important than dismutation (2), but this has not been fully investigated as yet.

No attempts have been made to isolate phosphorylated compounds intermediate between pyruvic acid and acetic acid. The removal of phosphorylated substances already in the enzyme preparation presents some difficulties. In this connexion it is interesting that Silverman & Werkman [1940] recently found that the conversion of pyruvic acid into acetyl methyl carbinol by extracts of *Aerobacter aerogenes* also required the presence of inorganic phosphate. Virtanen & Karström [1931] first observed that inorganic phosphate was required for the oxidation of pyruvic acid by bacteria.

SUMMARY

1. Cell-free extracts of *Bact. coli* do not contain carboxylase, but oxidize pyruvic acid to acetic acid and CO_2.

2. This pyruvic dehydrogenase system has been shown to comprise at least (*a*) a protein or proteins, (*b*) inorganic phosphate, (*c*) cocarboxylase and (*d*) a metal, possibly manganese or magnesium. Flavin adenine dinucleotide may also be a component of the system.

3. The oxidation of pyruvic acid in the presence of adenylic acid is probably coupled with the phosphorylation of the latter substance.

4. The extracts also decompose pyruvic acid under anaerobic conditions.

5. Acetaldehyde is oxidized only slowly, and could not be detected during the oxidation of pyruvic acid.

6. The extracts oxidize lactate rapidly in the presence of a carrier.

The author is grateful to Dr D. E. Green, Dr M. Stephenson and Dr R. P. Cook for their advice and interest in this work; and to Hebden Medical Fund of the University of Sydney for an assistance grant. The crushing mill used in this investigation was provided by a grant to this laboratory from the Medical Research Council.

REFERENCES

Bernhauer (1936). Garungschemisches Praktikum. Berlin: Springer.
Barron & Lyman (1939). *J. biol. Chem.* **127**, 143.
Booth & Green (1938). *Biochem. J.* **32**, 855.
Clift & Cook (1932). *Biochem. J.* **26**, 1288.
Clifton & Logan (1939). *J. Bact.* **37**, 523.
Cook (1930). *Biochem. J.* **24**, 1526.
Cook & Stephenson (1928). *Biochem. J.* **22**, 1368.
Dixon & Zerfas (1939). *Nature, Lond.*, **143**, 551.
Friedemann (1938). *J. biol. Chem.* **123**, 161.
Green, Herbert & Subrahmanyan (1940). *J. biol. Chem.* **135**, 795.
Krebs (1937, 1). *Biochem. J.* **31**, 661.
—— (1937, 2). *Biochem. J.* **31**, 2095.
Kruger & Tschirch (1930). *Ber. dtsch. chem. Ges.* **63**, 826.
Lipmann (1937). *Enzymologia*, **4**, 65.
— (1939, 1). *Nature, Lond.*, **143**, 281.
—— (1939, 2). *Nature, Lond.*, **143**, 436.
—— (1939, 3). *Cold Spring Harbor Symposia on Quantitative Biology*, **7**, 248.
Lutwak-Mann (1936). *Biochem. J.* **30**, 1405.
Mazza & Cimmino (1934). *Boll. Soc. ital. Biol. sper.* **9**, 1252.
Quastel & Wooldridge (1929). *Biochem. J.* **23**, 133.
Silverman & Werkman (1939). *Iowa State Coll. J. Sci.*, **13**, 365.
—— —— (1940). *Proc. Soc. exp. Biol., N.Y.*, **43**, 777.
Stephenson (1928). *Biochem. J.* **22**, 605.
Still (1940, 1). *Biochem. J.* **34**, 1177.
—— (1940, 2). *Biochem. J.* **34**, 1374.
Tikka (1935). *Biochem. Z.* **279**, 264.
Virtanen & Karström (1931). *Acta chem. fenn.* **7**, 17.

38. GLYOXALASE OF *BACTERIUM COLI*

By JACK LESLIE STILL

From the Biochemical Laboratory, Cambridge

(*Received 31 January 1941*)

Neuberg & Gorr [1925] observed that *Bact. coli*, both in culture and when dried, caused the conversion of methylglyoxal into lactic acid. Using yeast and *B. pasteur*, Lohmann [1932] investigated the properties of glyoxalase, the enzyme concerned, and determined the nature of its coenzyme or prosthetic group.

Cell-free extracts of *Bact. coli* prepared with the wet-crushing mill of Booth & Green [1938] contained an active glyoxalase. The following results were obtained with such extracts.

I. *Preparation*. *Bacterium coli commune* Escherich (National Collection of Type Cultures No. 86) was the source of the enzyme. Extracts were prepared as previously described [Still, 1941]. The extracts were used without heating or fractional precipitation, and retained their activity for 10 days without appreciable loss if kept at 0°.

II. *Methods*. The activity of the enzyme was measured in Warburg manometers by following the liberation of CO_2 from $NaHCO_3$-CO_2 buffer at *p*H 7·2. The methylglyoxal was prepared from 'Oxantin' by distillation in the presence of H_2SO_4 and estimated by the bisulphite fixation method of Clift & Cook [1932].

III. *Coenzyme*. The extract as prepared above showed very little activity, and this could be almost completely elminated by dialysis against running water for 12 hr. (Table 1).

Table 1. *Effect of dialysis on glyoxalase in extract*

Manometers containing 1·0 ml. extract, 0·4 ml. *M*/10 $NaHCO_3$, 0·4 ml. 0·23 % methylglyoxal. Total volume 3·2 ml.

	µl. CO_2/31 min.
Extract undialysed, no substrate	1·5
Extract undialysed, + substrate	25·0
Extract dialysed, no substrate	4·5
Extract dialysed, + substrate	2·0
No enzyme, + substrate	2·0

Addition of reduced glutathione more than restored the activity present before dialysis (Table 2).

Table 2. *Effect of GSH on activity of extract*

Manometers containing 1·0 ml. extract, 0·4 ml. *M*/10 $NaHCO_3$, 0·4 ml. 0·47 % methylglyoxal and 3·0 mg. GSH. Total volume 3·2 ml.

	µl. CO_2/20 min.
Extract undialysed, no substrate	3·0
Extract undialysed, + substrate	190·0
Extract dialysed, no substrate	3·5
Extract dialysed, + substrate	143·5
Extract dialysed, + substrate, no GSH	6·0

Oxidized glutathione in equivalent amounts did not replace GSH as coenzyme. However, an equivalent amount of cysteine brought about a considerable increase in the glyoxalase activity of the dialysed extract (Tables 3 and 4). Lohmann [1932] showed that with his yeast preparations no other SH-compound could replace GSH as coenzyme for glyoxalase. No explanation is offered for the action of cysteine in this instance.

Table 3. *Effect of concentration of GSH*

Manometers containing 1·0 ml. dialysed extract, 0·4 ml. $M/10$ $NaHCO_3$, 1·7 mg. phenylglyoxal. Total volume 3·2 ml.

GSH mg.	µl. CO_2/17 min.
–	26·0
0·25	60·5
0·5	70·5
1·0	85·0
2 0	96·0
3·0	88·0

Table 4. *Effect of GSH, GSSG and cysteine*

	Additions to manometers (ml.)				
Enzyme	0·8	0·8	0·8	0·8	0·8
$M/10$ $NaHCO_3$	0·4	0·4	0·4	0 4	0·4
Phenylglyoxal	0·4	0·4	0·4	–	0·4
GSH 2 mg./ml.	1·4	—	—	–	–
GSSG 4 mg./ml.	—	1·4	—	–	–
Cysteine-HCl 1 mg./ml.	—	—	1·0	1·0	–
Water	0·2	0·2	0·6	1·0	1·6
CO_2 output, µl./15 min.	202	9 5	127·5	3·0	33

IV. *Substrates.* Glyoxalase attacks several keto-aldehydes. The bacterial enzyme decomposed both methyl- and phenyl-glyoxals. Hayashi [1929] reported that *Bact. coli* attacked phenylglyoxal much less rapidly than methylglyoxal. This did not appear to be the case with the extracted enzyme.

Under suitable conditions the reaction proceeds to completion and the acid can be estimated. In one such experiment 540 µl. CO_2 were evolved, representing 92·5 % of theory. Lactic acid was estimated by the method of Friedemann & Graeser [1933]; 2·08 mg. were found, 2·17 mg. being the amount expected.

SUMMARY

Cell-free extracts of *Bact. coli* contain the enzyme glyoxalase, with properties essentially the same as those of the enzyme obtained from *B. pasteur* and yeast.

REFERENCES

Booth & Green (1938). *Biochem. J.* **32**, 855.
Clift & Cook (1932). *Biochem. J.* **26**, 1288.
Friedemann & Graeser (1933). *J. biol. Chem.* **100**, 281.
Hayashi (1929). *Biochem. Z.* **205**, 222.
Lohmann (1932). *Biochem. Z.* **254**, 332.
Neuberg & Gorr (1925). *Biochem. Z.* **162**, 490.
Still (1941). *Biochem. J.* **35**, 380.

39. ADENYLIC DEAMINASE ACTIVITY IN B_1-AVITAMINOSIS AND IN STARVATION

By GWEI DJEN LU and DOROTHY MOYLE NEEDHAM

From the Biochemical Laboratory, Cambridge

(*Received 15 January 1940*)

In our previous studies of carbohydrate metabolism in vitamin B_1 deficiency [Lu, 1939] it was shown that the high pyruvate level of the blood, though running closely parallel with the bradycardia, could not be regarded as directly responsible for it. Other possible causes therefore demanded consideration.

Two series of observations led us to examine the adenylic deaminase activity of pigeon breast muscle during B_1-avitaminosis. In the first place, Birch & Mapson [1936] comparing hearts of normal and B_1-deficient rats, had reported a decrease of about 20 % in the deaminase activity of the deficient hearts; 18 hr. after administration of the vitamin, the hearts of the recovered animals showed normal activity. These authors were in fact interested in the phenomenon from the point of view of the causation of the bradycardia of deficient animals, which resembles the bradycardia caused by injection of adenylic compounds into normal animals. Secondly, Bodnar & Karell [1932], using fluoride-poisoned brei from breast muscle of pigeons deficient in the B complex, had observed greater power than normal of accumulating phosphoric esters. Similar observations were made by Nagai [1932]. Since the presence of adenylic compounds is essential for ester formation, the greater production in the deficient animals might depend upon the higher concentration of adenylic compounds, and might be regarded as an indication suggesting loss of deaminase activity when the vitamin is absent. From the point of view both of the possible connection between adenylic acid and bradycardia, and of the disturbances in deficient muscle metabolism, it seemed of interest to study deamination of adenylic acid and phosphorylation of carbohydrate in skeletal and heart muscles of deficient animals.

Changes in adenylic deaminase activity in pigeon breast muscle

In order to measure the activity of the enzyme, a small portion of breast muscle (0·5–1·0 g.) was weighed, then ground in an ice-cold mortar with 10 vol. of ice-cold $M/10$ phosphate at pH 7, a few grains of Merck's quartz being added. After standing 30 min. at 0° for extraction of the enzyme, the extract was strained through muslin. In order to obviate uncertainties due to possible synthesis of added adenylic acid to adenylpyrophosphate (owing to any carbohydrate breakdown in the extract) the extract was always dialysed at 0° against 0·5 % KCl overnight before use; no change in volume took place during this dialysis. The activity of the extracts was tested in the following way: samples containing 1·0 ml. extract, 1·0 ml. phosphate buffer (0·16 M, pH 6·5), 1·0 ml. adenylic acid (neutralized, and containing 3 mg. adenylic acid) and 2·5 ml. water were mixed, and incubated for 20 and 40 min. at 37°. The NH_3 formation was estimated by the distillation method of Parnas & Heller, followed by nesslerization. The dialysed extracts were diluted before use in the tests, usually 1:10. The NH_3 production observed in the tests was therefore multiplied by 100 to obtain the production per g. muscle.

Preliminary experiments showed that the optimum *p*H lay at 6·5, and this *p*H was therefore always used.

In the earlier experiments, only one test of deaminase activity was performed on each bird, which was killed at the time of the removal of the muscle. These results, while definitely lower on the average in the deficient birds than in the normal, showed considerable variation within the two groups. The method of biopsy was therefore later adopted; a piece of muscle (30–60 mg.) was removed from the breast under ether anaesthesia, and the small wound sewn up. The birds recovered well. Not more than three operations were carried out on the same bird. Control experiments showed that pieces of muscle removed at intervals of some days from a bird kept on normal diet showed no variation in deaminase content; and further that the deaminase activity was the same whether the muscle was removed under ether, or without ether when the animal was killed. The weighed fragment of muscle was in each case extracted with 100 vol. of *M*/10 phosphate; this extract was used in the tests without further dilution.

The deficient diet usually consisted of thoroughly washed polished rice plus one drop of radiostoleum per day. The control birds received the same, together with 15 I.U. of vitamin B_1 each per day. When the deficient birds were to be recovered, they were usually put on to full stock diet (mixed pigeon corn) as well as receiving the amounts of B_1 recorded. The controls were put on to stock diet at the same time. In some cases, the effect of giving B_1 injections to the deficient birds, while continuing the rice diet, was tried. In some later experiments, as is pointed out in the appropriate places, a modified Carter diet was used. This consisted of 15 % Glaxo, 75 % rice starch, 5 % fresh butter and 5 % salts. It was supplemented by 0·5 g. autoclaved marmite, and one drop of radiostoleum every second or third day. This diet is not readily eaten by pigeons; but if they are provided with no other food, and if they receive B_1, they will accept it after a few days. All the birds in an experiment were therefore given B_1, until they were eating the diet well and maintaining weight. Then B_1 was removed from the diet of those destined for deficiency. The degree of this deficiency was judged (*a*) by onset of head retraction; this occurred fairly regularly in the earlier experiments, but much more variably later; (*b*) by heart rate; this symptom also is well known to be very unreliable in pigeons; (*c*) by the great loss of weight and weakness of the birds. With regard to the heart rate, Dr L. J. Harris, to whom we are indebted for much valuable advice, informed us that using the modified Carter diet he has succeeded in getting the heart rate reliably down, and up again with B_1; on this diet he found head retraction (which is probably a symptom of chronic B_1 deficiency) much rarer; the birds may die from heart failure in a shorter time and before head retraction comes on [Harris, 1939].

The curves in Figs. 1 and 2 show the results of the earlier series of experiments. It can be observed that the normals form a group with a considerably higher average than the deficients. The results for the recovered birds show an even wider scatter, and their average is intermediate between that of the other two groups.

In the later work, a series of experiments was done on each bird, muscle being removed at different stages of deficiency or during recovery.

Since B_1-avitaminosis leads to loss of appetite, great reduction in food intake and very serious wasting of the muscle, it was necessary to find out whether low diet plus B_1 led to change in deaminase activity of the muscle. Control birds were therefore used, paired with the deficient birds, so that each control received only as much food as the deficient partner had eaten on the previous day. The controls received 15 I.U. B_1 by injection per day. The importance of paired feeding is now becoming generally recognized in nutritional investigations [cf. McDonald & McHenry, 1939; Follis *et al.* 1940].

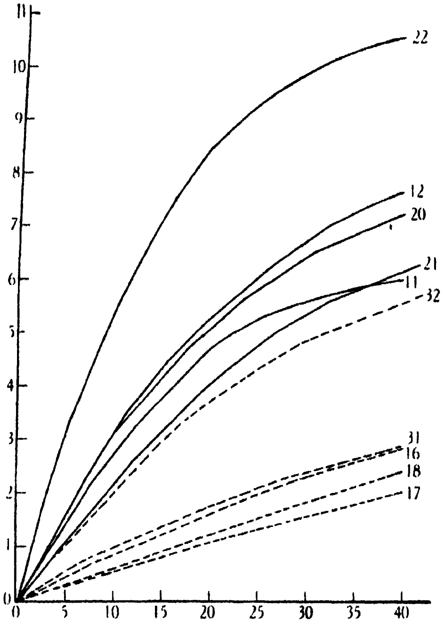


Fig. 1.

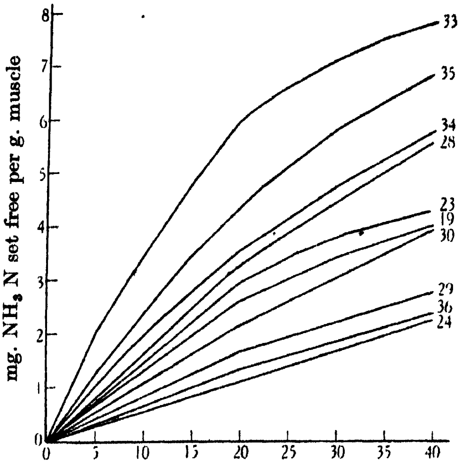


Fig. 2.

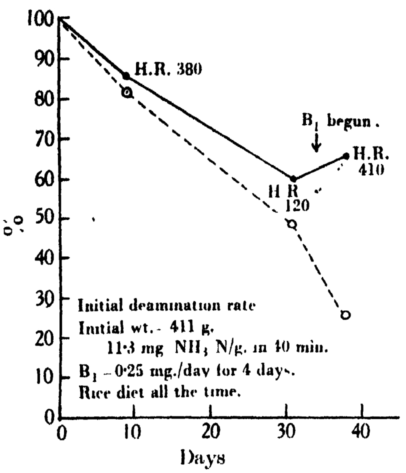
Fig. 1. All birds on polished rice diet. —— Normal. ---- Deficient.

Pigeon no.	Notes on deficient birds
16	Marked head retraction.
17	Marked head retraction. Heart rate 140.
18	Head retraction. Heart rate 195.
31	Marked head retraction.
32	Marked head retraction.

Fig. 2. All birds on polished rice diet till markedly deficient. Transferred to stock diet when B_1 was administered.

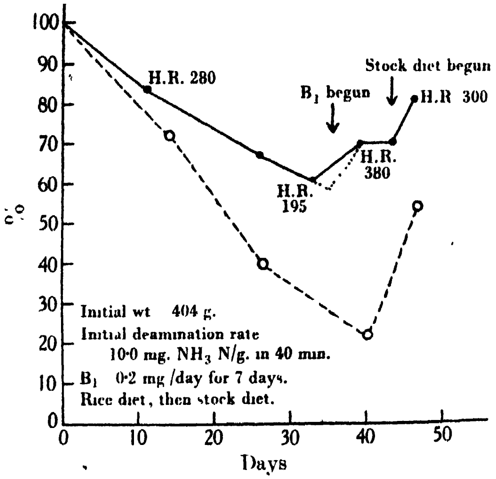
Pigeon no.	Notes on recovered birds.
28	Heart rate 240. 1 mg. B_1 per day given for 2 days and killed on the 3rd day. Heart rate 480.
29	Heart rate 245. 4 doses of B_1 given. Heart rate rose to 420 on the 2nd day; on the 5th day it was 220. Killed 5th day.
30	Heart rate 175. 1 dose of 0·3 mg. B_1. Killed next day. Heart rate 300.
19	Severe head retraction. 0·3 mg. B_1 per day for 4 days. Killed 5th day. Heart rate 285.
23	Received 2 mg. B_1. Killed next day. Heart rate 240.
24	Heart rate 240. Received 1 mg. B_1 each day for 5 days. Killed 6th day. Heart rate 450.
34	Head retraction. 0·3 mg. B_1 each day for 2 days. Killed 3rd day. Heart rate 280.
35	Received 0·3 mg. B_1. Interval of 1 day. Then 1 mg. a day for 2 days. Killed next day. Heart rate 180.
33	Marked head retraction. 1 mg. B_1 per day for 2 days. Killed 3rd day. Heart rate 310.
36	Heart rate 154. 0·3 mg. B_1. Interval of 2 days. Then 1 mg. per day for 4 days. Killed next day. Heart rate 430.

The results show in every case a marked fall in deaminase activity in the same bird when examined before and after production of avitaminosis (Figs. 3–8 and 10–11). In every case of semi-starvation plus B_1 administration, a marked fall was also found; this fall was not so great as in the deficient birds but neither



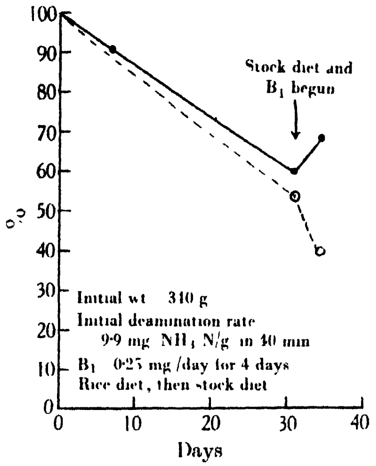
Initial deamination rate
Initial wt. - 411 g.
11·3 mg NH_3 N/g. in 40 min.
B_1 - 0·25 mg./day for 4 days.
Rice diet all the time.

Fig. 3. No. 38.



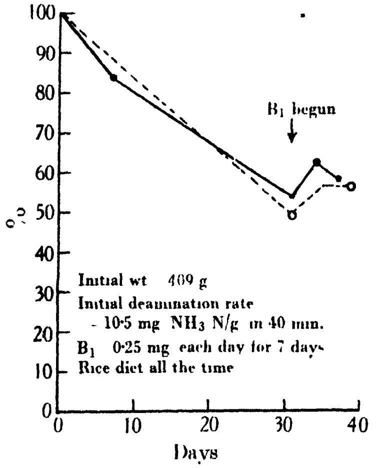
Initial wt 404 g.
Initial deamination rate
10·0 mg. NH_3 N/g. in 40 min.
B_1 0·2 mg/day for 7 days.
Rice diet, then stock diet.

Fig. 4. No. 42.



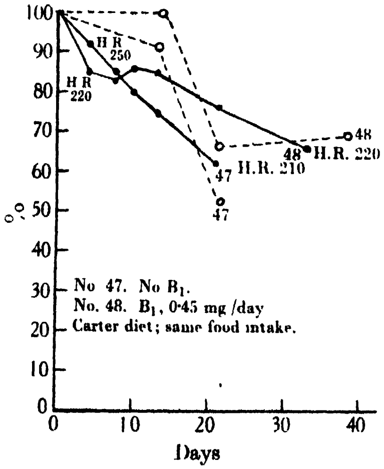
Initial wt 310 g
Initial deamination rate
9·9 mg NH_3 N/g in 40 min
B_1 0·25 mg/day for 4 days
Rice diet, then stock diet

Fig. 5. No. 43.



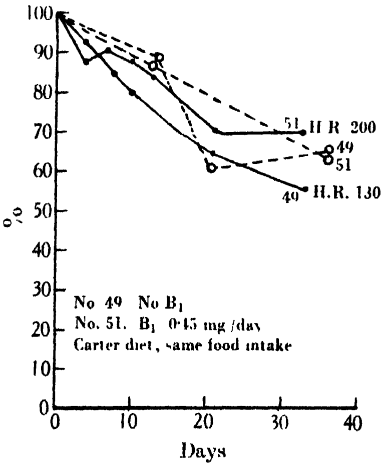
Initial wt 409 g
Initial deamination rate
- 10·5 mg NH_3 N/g in 40 min.
B_1 0·25 mg each day for 7 days
Rice diet all the time

Fig. 6. No. 44.



No 47. No B_1.
No. 48. B_1, 0·45 mg/day
Carter diet; same food intake.

Fig. 7.



No 49 No B_1
No. 51. B_1 0·45 mg/day
Carter diet, same food intake

Fig. 8.

In Figs. 3–11 — indicates body weight; - - - indicates deamination rate;
H.R. indicates heart rate.

was the loss of weight so great, in spite of the fact that the food intake was the same (Figs. 7–11).

This suggests that the loss of deaminase activity depends rather on the starvation condition than on the absence of B_1. The results of experiments on recovery substantiate this view (Figs. 3–6, 9, 10). For although return of deaminase activity is sometimes seen in these experiments after administration of B_1, it occurs to a marked extent only when the animal is rapidly recovering weight. If for example B_1 is given but the rice diet is continued, there is usually a rise in heart rate and general improvement in the condition of the animal; but after an initial rise the weight may remain stationary or fall slightly again. If, on the other hand, the administration of B_1 is accompanied by a change to the stock diet, there is rapid increase in weight, which may return to normal in about 4 days. It is only under the latter conditions that marked recovery of deaminase activity has been observed, and even under these conditions there

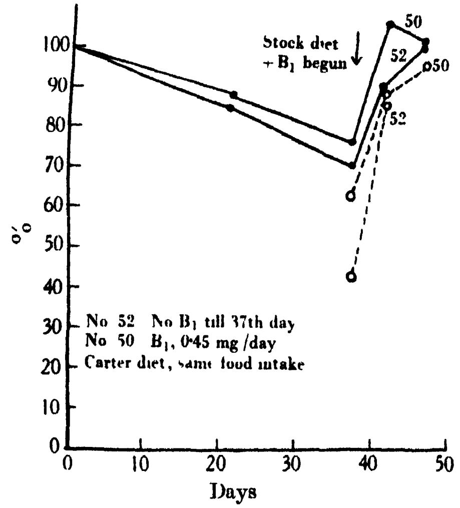


Fig. 9.

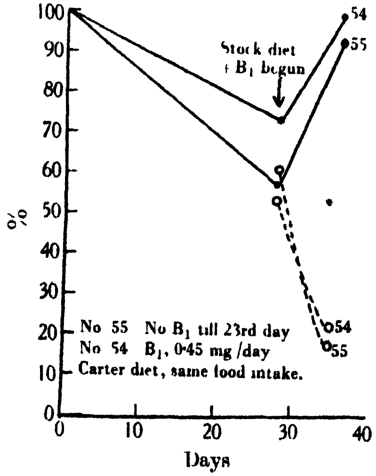


Fig. 10.

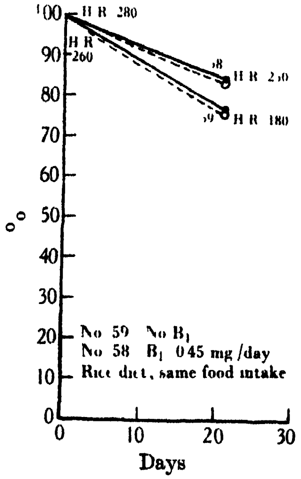


Fig. 11.

were cases where the deaminase activity continued to fall while the muscle weight was being rapidly regained. This question is further discussed later.

In a few cases the B_1 content of the muscle and liver tissue was estimated by the thiochrome method.

	Liver µg. B_1 per g.	Muscle µg. B_1 per g.
No. 49 (killed in deficient state)	0·06	<0·03
51 (semi-starved but receiving B_1)	2·3	1·1
56 (semi-starved but receiving B_1; last dose day before death)	3·4	2·5

These results show that, in spite of the great wastage of tissue going on, the animals receiving B_1 retain it in their organs.

Changes in phosphorylation activity in pigeon breast muscle

The results of Bodnar & Karell [1932] on increased esterification of phosphate in B_1-deficient muscle were confirmed. We used the same conditions as theirs, except that the samples were kept at 37° for 40 min. instead of for some hours at room temperature. 1 g. brei was treated with 0·5 ml. 1 % Na_2HPO_4, 0·5 ml. 2 % NaF and 15 mg. glycogen. The mean of eight experiments on normal birds gave a value of 0·72 mg. P esterified in 40 min. (limits 0·4–1·0), while the mean for nine deficient birds was 0·98 mg. (limits 0·7–1·3). These differences are not so large or so constant as those found by Bodnar & Karell—their averages were 0·70 mg. for normal and 1·25 mg. for deficient animals.

In the brei inorganic P is disappearing, both by the Parnas esterification of glycogen with formation of hexosemonophosphate, and the 'coupled' esterification via adenylic acid with formation of hexosediphosphate. Some experiments were done to see whether the phosphorylase system showed different activities in the two cases. Muscle extract was made by crushing the muscle with an equal volume of ice-cold water; in order to obtain esterification by the phosphorylase system alone two methods were adopted:

(1) The extract was allowed to stand 24 hr. before use.

(2) The fresh extract was incubated with $M/250$ iodoacetic acid for 15 min. before addition of substrate, to bring about inhibition of the 'coupled' mechanism.

In both cases, to 1 ml. of extract were added 0·5 ml. 0·33 M phosphate at pH 7·0 and 15 mg. glycogen.

The average result for five extracts from normal birds by the first method was 0·58 mg. P esterified per ml. in 45 min.; for six deficient birds the average was 1·05 mg. By the second method the average for four normal birds was 0·66 mg., for five deficient birds 0·89 mg.

It thus appears that phosphorylase activity is enhanced to some extent in the B_1-deficient animals. In this connexion it is interesting that although muscular weakness and dystrophy exist also in vitamin E deficiency, the phosphate esterification is there distinctly decreased [Lu *et al.* 1940].

Adenylic deaminase and vitamin B_1 in vitro

Before it became likely that the connexion between the enzyme and the vitamin was an indirect one, we made some tests to find whether addition of the vitamin or cocarboxylase to the deficient extract would restore the deaminase activity to normal. But although we tried many experimental variations, we were never able to observe any such activating effect.

Changes in deaminase activity in rat leg muscle

Similar experiments to those on the pigeon breast muscle were carried out on rat leg muscle. In each case all the muscles of one leg were removed, chopped and well mixed, before a portion was weighed out. The results are given in Table 1.

Table 1. *Change in deaminase activity of normal and deficient rat leg muscle*

	No.	Heart rate	Deamination mg. NH_3 N set free by 1 g. muscle in 40 min. at 37°
Normal	II	—	2·24
	XIII	534	2·85
	XIV	500	2·25
	XV	540	3·60
	XVI	520	3·70
	XVII	540	3·35
	XVIII	570	3·63
	XXVII	490	2·17
	XXVIII	520	2·37
		Average	2·90
Deficient	III	—	3·36
	IV	—	2·35
	V	375	2·34
	VI	300	2·09
	VII	385	2·27
	VIII	320	2·03
	XIX	—	2·12
	XX	—	2·40
		Average	2·37
Recovered	IX	475	2·85
	X		1·95
	XI	490	1·89
	XII	530	3·03
		Average	2·43

It is obvious that the decrease in deaminase activity is not nearly so marked as in pigeon muscle; on the average it does not exceed 20 %. It is to be observed that even this small difference between the normal and the deficient depends on the high values in rats nos. XV–XVIII, all of which belonged to one group.

Adenylic deaminase activity in rat heart muscle

In a number of the rat experiments just described, the deaminase activity of the hearts was also tested, and to our surprise, no significant difference between the three groups could be detected. A series of experiments was therefore carried out, using similar conditions to those of Birch & Mapson [1936], to whom we are indebted for information about their experimental details. The heart was weighed and ground with quartz and 10 vol. of ice-cold water in a cooled mortar. After extracting at 0° for 30 min., the extract was strained off through muslin and allowed to stand at room temperature for 2 hr. in order that any glycolysis in the extract might be completed. 1 ml. of the extract was then mixed with 1·2 ml. $M/6$ phosphate buffer pH 7·0, 0·2 ml. neutralized solution containing 6 mg. adenylic acid, and 3·6 ml. water. The samples were incubated at 37° for 20 and 45 min. (Table 2). The difference is only 7 %.

Table 2. *Changes in deaminase activity of normal and deficient rat heart muscle*

	No.	Heart rate	Deamination mg. NH_3N set free by 1 g. heart in 45 min. at 37°
Normal	33	550	0·68
	34	515	0·62
	42	500	0·73
	43	520	0·71
	46	480	0·64
	47	500	0·62
	54	510	0·52
	53	480	0·60
			Average 0·64
Deficient	35	410	0·64
	36	390	—
	37	380	0·77
	38	390	0·65
	39	314	0·65
	40	130	0·74
	41	220	0·73
	44	320	0·66
	45	350	0·70
	55	280	0·68
	52	300	0·76
			Average 0·69

The effect of ions on the adenylic deaminase

While this work was in progress a paper by Conway & Cooke [1939] appeared in which the powerful effect of certain anions, notably phosphate and carbonate, in inhibiting the activity of the deaminase at low concentrations of substrate was described. It seemed necessary, therefore, especially as our substrate concentrations were low (0·06 %), to include examination in citrate buffer in determining the activity of the enzyme. Extra samples were set up in which the *M*/30 phosphate buffer was replaced by *M*/20 citrate. With pigeon muscle it was found that deamination is about 3·5 times as fast in citrate buffer under our conditions; with rat muscle, about 8·5 times as fast. The effect of the citrate buffer appeared to the same extent with both normal and deficient muscle extracts. Conway & Cooke suggest that the effect of the phosphate ion may be explained by assuming adsorption of the anion on the enzyme with exclusion of the adenylic acid, which would agree with the diminished inhibitory effect found by them with increasing substrate concentration in rabbit muscle extract. Similar results were obtained by us on rat muscle extract. In citrate buffer, using 1 : 1250 dilution of muscle extract, at 0·06 % adenylic acid concentration, 9·28 mg./g. NH_3-N were set free in 10 min.; with 10 times the substrate concentration, 0·6 %, the NH_3 formation was approximately doubled—21·0 mg./g. In phosphate buffer, on the other hand, increasing the substrate concentration ten-fold led to a nearly twenty-fold increase in deamination. At 0·06 % adenylic acid, the NH_3-N set free was 1·1 mg./g. in 10 min.; at 0·6 % substrate, 18·65 mg./g.

It may be noticed that in citrate buffer, where presumably the enzyme is maximally active, there is little difference between the deamination rates of pigeon and rat muscles; in the rat muscle extract the enzyme shows a greater degree of inhibition by phosphate.

The rates of deamination in rat heart muscle were also compared in phosphate and citrate buffers. Very little difference was found.

	NH_3-N mg./g.	
	In phosphate	In citrate
Normal heart	0·68	0·74
Deficient heart	0·62	0·58

The bulk of the heart consists, of course, of ventricle. According to Conway & Cooke [1939] ventricular tissue in the rabbit contains an adenosine deaminase but little if any true adenylic deaminase, although the latter is present in the auricular tissue. The adenosine deaminase was found by them not to be inhibited by phosphate. The fact therefore that the rat heart shows little difference of activity in different buffers suggests that here too an adenosine deaminase may be concerned. Conway & Cooke propose two methods whereby it may be tested whether adenylic acid is directly deaminated, or only after initial dephosphorylation.

(1) Adenylic deaminase of animal tissues seems to be specific for muscle adenylic acid, but if the deamination is indirect, by means of adenosine deaminase, the rates of deamination of yeast and muscle adenylic acids will be the same (assuming the rates of dephosphorylation to be the same). This method was tried on rat heart, but it was found that yeast adenylic acid was practically untouched. This result is not conclusive for the presence of true adenylic deaminase, as it may only mean that the rates of dephosphorylation differ in this case.

(2) The NH_3 set free after addition of adenosine to saturate the adenosine deaminase may be compared with the amounts set free after addition of adenosine and adenylic acid together.

The following experiment was performed. The extract was made as usual from normal hearts. The adenosine solution contained 12·5 mg./ml.; the adenylic acid solution 30 mg./ml.; the buffer was *M*/6 phosphate, *p*H 7·0.

	1	2	3	4	5
Extract	1·0	1·0	1·0	1·0	1·0
Buffer	0·6	0·6	0·6	0·6	0·6
Adenylic acid	0·2	0	0	0 2	0·2
Adenosine	0	0·4	0·8	0·4	0·8
NH_3 formation, mg. N/g. tissue in 20 min.	0·029	0·057	0·054	0·054	0·054

These results show that adenosine deaminase is present and saturated with substrate under the conditions used, for doubling the concentration of adenosine leads to no increase in NH_3 formation. That a separate enzyme for deamination of adenylic acid is absent, is shown by the fact that addition of adenylic acid to the system has no effect on the liberation of NH_3.

DISCUSSION

In this work it has been established that during the inanition accompanying vitamin B_1 deficiency in the pigeon, skeletal muscle suffers a loss in adenylic deaminase which may be as great as 75 %. In two or three cases it was even 90%. That the changes cannot be due to changes in water content of the muscle is shown by the results of Roche [1924], who found the water content of normal pigeon breast muscle to be 74·8 %, of avitaminotic birds (on polished rice diet) 73·6 % and of birds in inanition 75·3 %.

The facts that injection of vitamin B_1 leads to no rapid increase in deaminase activity, that the fall occurs on a low diet even if vitamin B_1 is injected and that vitamin B_1 has no activating effect on the enzyme of the deficient extract *in vitro*, all indicate that the vitamin is not playing here any sort of coenzyme role, as its pyrophosphate does in the oxidation of pyruvic acid.

It may be interesting to compare the effect of vitamin B_1 deficiency on deamination with its effect on respiration. Roche [1924] found that respiration of the isolated muscle tissue was the same in normal, deficient and starved birds, and he did not observe any fluctuations due to rice in the crop, etc. Galvao & Pereira [1937] found that breast muscle tissue from vitamin B_1-deficient pigeons may have a very low respiration, even after administration of B_1 to the bird. On the other hand, the respiration of the muscle from the recovered animals might rise to normal, and this return seemed to be correlated with the ingestion of food, or with the fact that the birds, in becoming deficient, had not lost excessively in weight. The suggestion is that the intensity of tissue respiration observed depends not only on the presence of the vitamin, but also on availability of substrate.

The same conclusion is reached by Lipschitz *et al.* [1938] in their work on the pyruvate oxidation by brain, kidney and liver, in vitamin B_1-deficient chicks. They found that the ability of kidney, liver and brain tissue from polyneuritic birds to oxidise added pyruvate was impaired. The feeding of glucose to the deficient birds led to a rise in the power of the liver to remove pyruvate, though normal values were not reached. The liver and kidney tissue from fasted birds also showed a decreased power to remove pyruvate, and the oral administration of glucose to the fasted birds led to restoration of the ability of the liver tissue to remove pyruvate. Thus it seemed that both with polyneuritic and fasted birds, the provision of some other substrates (derived from glucose) was involved in the pyruvate removal. In the fasted birds, the provision of these substrates seemed to be the only factor necessary; with the polyneuritic birds, complete restoration of the power to remove pyruvate was not attained with glucose only.

In the case of deamination it seems that the explanation of the loss of deaminase activity is not loss of coenzyme (see above) or lack of metabolites, for in all cases the activity is tested in greatly diluted dialysed extracts; the suggestion arises that the enzyme is actually lost from the tissue. In the great loss of material that goes on from the muscle, all the protein fractions may not be involved in exact proportion to their concentration in the muscle: it would not be surprising if, in the substance broken down and removed, some enzymes were involved more than others. That some enzymes escape destruction to a disproportionate degree is suggested by Nagai's finding [1932] that glycerophosphatase activity is greater by about 20 %, and hexosediphosphatase activity by about 40 % in the deficient muscle. But phosphate metabolism in a muscle brei is so complex that a number of factors may be influencing the accumulation of phosphate in these cases just as they may be influencing esterification of phosphate in the phosphorylation experiments mentioned earlier.

If the protein of the deaminase enzyme has to be resynthesised after recovery, the conditions governing its reappearance in full activity may be very complex. The muscle might increase greatly in weight and in protein content, without proportionate formation of the enzyme, and this state of affairs might account for the cases (such as 54 and 55, Fig. 10), where, after administration of the vitamin and stock diet, there is rapid increase in weight accompanied by still further fall in deaminase activity.

The function of the adenylic and adenosine deaminases in general, and their relation to the regulation of heart beat in particular, are obscure. Their function, especially that of the very active adenylic deaminase in skeletal muscle, has often been regarded as one of detoxication, and Birch & Mapson [1936] regarded even a 20 % decrease in the activity of the heart enzyme as a possible explanation of the bradycardia of B_1 deficiency. As we have found, however, this fall in heart deaminase activity does not always accompany B_1-avitaminosis, even when the bradycardia is as well marked as usual; and, moreover, the heart rate may return to normal while the deaminase of the skeletal muscles does not do so (see e.g. Figs. 3 and 4).

Conway & Cooke [1939] have pointed out that in spite of the deaminases of the tissues, an adenine nucleotide (other than muscle adenylic acid) exists in blood plasma, to the extent of about 5-10 mg./100 ml.; red blood cells contain adenylpyrophosphate and adenosine, and are freely permeable to the adenylic acid which is probably formed there. There is no evidence as to the concentrations of adenylic acid compounds normally reaching the sensitive sites of the heart, or what increase in concentration is necessary at these sites for the effects of bradycardia to be produced.

Conway & Cooke have remarked that the effective deamination rate in skeletal muscle is 500-1000 times that in any other tissue. NH_3 is also formed in skeletal muscle from adenylic acid 20 times as fast as it is from adenosine by the tissues with the most active adenosinase (appendix, jejunum). It seems that considerable quantities of adenine nucleotides must escape from the intestine into the blood; and if this is the case it becomes difficult to regard the skeletal deaminase as a mechanism to protect the rest of the body from effects of adenylic acid. If the adenylic deaminase has some function within the muscle itself, this is at present unknown.

SUMMARY

1. Muscle tissue of animals in vitamin B_1 deficiency shows a diminution of the activity of adenylic deaminase. This is especially marked in pigeon breast muscle, less so in rat leg muscle. Differences between these animals in the absolute levels of their deaminase activities may be largely due to ionic inhibition effects as discussed in the text. Rat heart muscle deaminates adenylic acid indirectly. The latter is first dephosphorylated, and then NH_3 formation is caused by action of adenosine deaminase. No difference was observed in rate of NH_3 liberation between normal and deficient heart muscles.

2. Decrease in the adenylic deaminase activity is accompanied by some increase in phosphorylase activity in the deficient muscle.

3. Paired feeding experiments with inanition unaccompanied by vitamin B_1 deficiency show, however, that the diminution of deaminase activity is primarily connected with the starvation, and only secondarily with vitamin deficiency. The return to normal is slow, requires normal food intake and cannot be brought about by vitamin B_1 alone.

4. Bradycardia, on the other hand, gives place rapidly to normal heart rate as a result of administration of B_1 alone. It seems unlikely, therefore, that the bradycardia is due to accumulation of adenylic acid following the decreased deaminase activity.

The thanks of the authors are due to Dr Leslie J. Harris for much valuable advice and assistance; to Mr Ying-Lai Wang for carrying out thiochrome estimations; to Dr H. Lehmann for advice on biopsy technique; and to Miss R. Leader, Mr H. Mowl and Miss L. Gordon for technical assistance.

REFERENCES

Birch & Mapson (1936). *Nature, Lond.*, **138**, 27.
Bodnar & Karell (1932). *Biochem. Z.* **230**, 233.
Conway & Cooke (1939). *Biochem. J.* **33**. 479.
Follis, Day & McCollum (1940). *J. Nutrit.* **12**, 181.
Galvao & Pereira (1937). *Hoppe-Seyl Z.* **245**. 19.
Harris (1939). *Chem. Ind.* **58**, 472.
Lipschitz, Potter & Elvehjem (1938). *J. biol. Chem.* **123**, 267.
Lu (1939). *Biochem. J.* **33**, 774.
— Emerson & Evans (1940). *Amer. J. Physiol.* **129**, 112.
McDonald & McHenry (1939). *Amer. J. Physiol.* **128**, 608.
Nagai (1932). *J. Biochem., Japan*, **16**, 359.
Roche (1924). *Arch. int. Physiol.* **24**, 413.

40. THE CYSTINE AND METHIONINE IN CERTAIN SPECIES OF GRASSLAND HERBAGE

By ALEXANDER MARTIN SMITH AND TUNG WANG

From the Chemistry Department, Edinburgh and East of Scotland College of Agriculture

(*Received 3 March 1941*)

It would appear from recent investigations that both of the amino-acids containing sulphur are to be regarded as essential in animal nutrition, so that it is important to have data on the amounts of these acids in animal foodstuffs. Grass is the most important source of protein to the animal, and the object of the present investigation was to determine the cystine and methionine in a few of the more important species of grassland herbage. Most of the work with the grasses has been carried out by Chibnall and his co-workers, who have made fairly comprehensive analyses of the protein preparations of the leaves of many plants.

EXPERIMENTAL

Material examined. Attention was confined to four species, viz. white clover, *Trifolium repens*; cocksfoot (Danish), *Dactylis glomerata*; perennial rye-grass (Ayrshire), *Lolium perenne*; and timothy (Scotch), *Phleum pratense*. Samples were taken at different stages of growth from different places during two seasons. Most of the farms were in late districts and in rather exposed areas, so that there was not much growth of grass before May when the first samples were collected. One farm was situated on deep peat which had recently been reclaimed by drainage and had received liberal dressings of lime and fertilizers. The other farms were on mineral soils varying in texture from light to heavy loams and in *p*H value from about 5 to 7·5. The readily available potassium and phosphorus varied from very low to moderately good values. A few experiments with fertilizers were incorporated in the plan of the work, but, as is shown below, the results were relatively unimportant. In some cases the samples were obtained from plots of pure species, but usually the herbage was mixed and the individual species were separated by hand. The plants were cut about 1 in. above ground level with a knife and the fresh material was transported to the laboratory in waterproof bags and examined immediately.

Separation of protein. The preparation of a reasonably pure protein from the leaves of plants has engaged the attention of many workers and the question is fully dealt with by Chibnall [1939]. In the present investigation, it was decided to employ the method of extraction using a slightly alkaline buffer solution as suggested by Lugg [1939]. Between 300 and 400 g. of the fresh plant material were disintegrated twice in a mincing machine with about three times that quantity of a solution of borax containing 1·1 g. boron per litre and of *p*H 9·2. The cell-wall debris was removed by squeezing the pulp in a linen bag and the colloidal extract was filtered through paper on a Büchner funnel. The

pH of the filtrate was brought to about 4·5 by the addition of 4 ml. 2N H_2SO_4 per 100 ml. when the protein settled out quite quickly. Next day the supernatant liquid was siphoned off and the precipitate was washed twice with water. The protein was then separated by centrifuge, washed four times with absolute alcohol and twice with methylated ether and then extracted for 20 hr. in a Soxhlet apparatus with light petroleum (B.P. 40–60°). This final extraction was found to be necessary to remove traces of ether-soluble material which interfered with the subsequent determination of methionine. The protein preparation was finally dried over P_2O_5 in a vacuum.

Protein preparations

Yield and total nitrogen. The percentage of N in the protein preparations was rather irregular. It varied from 10 to nearly 14 % for white clover, from about 9 to 14 % for cocksfoot, from 6 to 13 % for rye-grass and from 8 to about 13 % for timothy. The success of the extraction naturally depends upon the thoroughness of disintegration and probably upon the nature of the protoplasm. The clover yielded the most protein, the recovery of the N in the original sample varying from 24 to 43 %. Cocksfoot on the other hand yielded the least protein, the preparation accounting for only 15 to 34 % of the total N. Within each species, more protein was usually obtained from the young and aftermath samples than from the samples of older material. The results are summarized in Table 1, in which the samples have been grouped according to stage of growth rather than to date of sampling.

Table 1. *N and amino-N (after hydrolysis) in fat-free protein preparations from white clover (W.C.), cocksfoot (C.), perennial rye-grass (R.) and timothy (T.)*

	% of N in protein preparation				N in protein preparation as % of total leaf-N				% total N in protein preparation as amino-N after hydrolysis			
Nature of sample	W.C.	C.	R.	T.	W.C.	C.	R.	T.	W.C.	C.	R.	T.
Young	10·5	12·7	10·0	8·0	38	29	21	*10	66	61	60	58
	13·8	—	—	—	*6	—	—	—	65	—	—	—
	12·8	—	—		37	—	—	—	68	—	—	—
Flowering or heading	10·2	10·6	10·4	10·7	*16	24	24	31	71	60	61	64
	10·0	11·0	12·0	10·8	31	26	22	25	59	57	66	62
	10·9	12·9	9·6	—	28	22	25	—	63	62	68	—
	—	14·1	12·8	—	—	26	24	—	—	63	60	—
Old	†10·2	9·7	6·0	11·1	30	20	25	*20	73	72	53	67
	11·7	9·9	8·6	—	31	17	22	—	68	63	63	—
	13·2	11·6	—	—	24	21	—	—	72	63	—	—
Aftermath	11·1	11·0	†8·1	†12·5	34	26	30	29	66	64	57	57
	11·9	11·2	10·2	8·5	27	21	32	33	68	64	59	57
	—	10·1	11·0	—	—	23	34	—	—	61	59	—
	—	†8·6	10·3	—	—	20	28	—	—	70	57	—
	—	10·9	10·7	—	—	27	27	—	—	62	62	—
	—	10·4	—	—	—	15	—	—	—	64	—	—
Grazed	13·8	9·5	10·8	8·6	43	34	26	31	61	63	58	62
	13·0	11·6	12·5	11·8	32	32	29	34	70	63	63	62
	—	—	13·1	—	—	—	24	—	—	—	58	—
	—	—	12·3	—	—	—	25	—	—	—	62	—
Average	11·8	11·0	10·5	10·3	—	—	—	—	67	63	60	61
Ash content	4·8	6·8	7·6	4·3	—	—	—	—	—	—	—	—

* A portion of protein lost during manipulation.
† Samples subjected to determination of ash.

The figures for the N recovered as protein are affected, of course, by the inevitable losses of material during extraction and consequently give only a general idea of the extent of the extraction. In the case of two samples of rye-grass, the residue from extraction was re-extracted and the protein precipitated and prepared in the usual manner. The amount obtained was very small, being only about a fifth of the first preparation, but there was enough to make determinations of total N and amino-N after hydrolysis. A comparison of these figures for the first and second preparations indicates that there is no striking difference between the protein obtained in the first extraction and that left in the residue and lends support to certain observations made by Chibnall [1939].

	Total N in protein preparation		Amino-N after hydrolysis as % of total N	
Sample	1st extract	2nd extract	1st extract	2nd extract
1	11·7	10·4	63	63
2	11·9	9·8	57	57

Ash. The total ash was determined in only one sample of each species and the results varied from 4·3 to 7·6 %.

Amino-N. The total amino-N was determined by the Van Slyke [1915] gasometric method using a micro-apparatus. Some preliminary tests were carried out with glycine to find the time of shaking required to give a maximum yield of amino-N. Glycine gives high but consistent results and a maximum recovery of 111·8 % was obtained with 8 min. shaking at about 300 r.p.m.; this figure is similar to that obtained by Levene & Van Slyke [1912], and a period of 8 min. shaking was adopted for further work. Five other amino-acids were examined; a small quantity of each was dissolved in water (a little dil. HCl was used with cystine and tyrosine) and made up to a definite volume from which 2 ml. were taken for each test. The average results are given in Table 2 and compared with those obtained by Van Slyke [1911] for the same compounds.

Table 2. *Average percentage recovery of amino-N*

	Arginine	Cystine	Histidine	Lysine	Tyrosine
Present investigation	102·3	108·5	103·0	86·9	102·4
Van Slyke [1911]	99·8	107·4	98·2	95·4	102·1

It will be observed that the results for arginine, histidine and tyrosine are not far removed from the theoretical values, whilst that for cystine is much higher and that for lysine rather low. The amounts of glycine, cystine and lysine in grass proteins are relatively small, however [Chibnall, 1939], so that the error in the total amino-N estimated by the above procedure is unlikely to be serious.

The grass proteins were hydrolysed with acid; a few preliminary experiments showed that a maximum percentage of amino-N was obtained when about 1 g. of the preparation was boiled with 25 ml. of 3*N* HCl for 8 hr. The yield of amino-N was not affected by the previous addition of known amounts of amino-acid to the protein preparation. Figures for the proportion of amino-N in the different preparations are included in Table 1. There are no important differences between the protein preparations of the four species with regard to the percentages of total amino-N; the figures for clover vary from 59 to 73, for cocksfoot from 57 to 72, for rye-grass from 53 to 68, and for timothy from 57 to 67. It will be observed, however, that the amount of amino-N tends to increase as the plant becomes ripe—a fact which is particularly noticeable when a comparison is made between samples taken from the same farm. Generally speaking, it may be said that the amount of amino-N in the hydrolysed samples of all four species reached a maximum during July.

Manurial treatment. The effects of manurial treatment were rather irregular. Lime increased the amino-N in both cocksfoot and rye-grass, but with slag the results obtained for cocksfoot were the reverse of those obtained for rye-grass. A late application of ammonium sulphate, three weeks before cutting, produced a considerable decrease (about 12 %) in the amino-N of rye-grass protein, although the total N in the grass was increased by about 40 %.

Sulphur-containing amino-acids

The method used in the present investigation was based on the procedure devised by Baernstein [1936] and involved hydrolysis with HI.

Apparatus. A few modifications were made of the apparatus used by Baernstein. The digestion flask was connected to the condenser by a ground joint which seemed to be tight enough without the mercury seal suggested by Kassell & Brand [1938]. The absorbers were connected in series by rubber stoppers and tubing. The first absorber contained 10 ml. of a 1 % suspension of red phosphorus in distilled water; the outlet of this tube was packed with glass wool to prevent the phosphorus from being carried over. The second absorber contained 10 ml. of a solution containing 20 % $CdCl_2$ and 20 % $BaCl_2$ to absorb H_2S and SO_2; the third contained 10 ml. of saturated $HgCl_2$ to remove phosphine from the KH_2PO_2 added to the HI; the fourth contained 10 ml. of 10 % potassium acetate in glacial acetic acid with six drops of bromine. This solution absorbs the methyl iodide produced from the methionine and the fifth absorber contained 5 ml. of the same solution to ensure complete absorption of the volatile iodide. The HI was purified by double distillation; its specific gravity was 1·7 and 1 % KH_2PO_2 was added to prevent formation of free iodine.

Hydrolysis. About 0·5 g. of the protein preparation was placed in the digestion flask (capacity about 50 ml.), which was then filled with nitrogen gas previously passed through a solution of 5 % $AgNO_3$ and concentrated H_2SO_4. A few small pieces of unglazed porcelain, 10 ml. of HI and about 0·02 g. of KH_2PO_2 were then added and the flask was connected to the condenser, in which the water was maintained at about 50°. The absorption train was then assembled and attached to the condenser. The digestion flask was immersed in an oil bath at 150° and the first three absorbers in water at 50–60°. During hydrolysis, a stream of nitrogen was passed through the apparatus at a constant rate controlled by a pinch cock on the side tube of the digestion flask. Maximum values were obtained when the boiling and aeration were continued for 5–6 hr. for pure protein and for 7–8 hr. for grass protein.

Cystine. At the end of the digestion, the absorption train was disconnected from the condenser, 0·02 g. of KH_2PO_2 was added to the digestion flask and, with suction from a pump at the condenser outlet, the digest was concentrated to about 3 ml., the stream of nitrogen being continued meantime. After the digest had cooled in a current of nitrogen it was made up to 25 ml. with 4 % HCl. 10 ml. were pipetted into a conical flask filled with nitrogen and a few ml. of $N/100$ $KIO_3.HIO_3$ were added until the solution was definitely orange in colour. The excess iodine was titrated with $N/100$ $Na_2S_2O_3$, using starch as an indicator.

Methionine. The methionine, which has been converted during the hydrolysis into the lactone of homocysteine, was now determined in the same solution. A few drops of bromothymol blue were added together with one drop of octyl alcohol and a quantity of freshly prepared $0·04\,N$ $Na_2S_4O_6$ so as to give, with the tetrathionate produced in the cystine titration, an excess of 2·5 ml. 1 ml. of

0·04 N $Na_2S_4O_6$ is equivalent to 6 mg. of methionine and the amount of methionine present was already estimated in the volatile iodide determination described below. The flask was fitted with a rubber stopper, carrying two tubes, and filled with nitrogen gas. It was then evacuated and about 2 ml. of concentrated ammonia were drawn in from a burette, inserted through one of the tubes, to make the solution definitely alkaline. The flask was again evacuated and immersed in warm water at 40° for 15 min. It was again filled with nitrogen and the connecting tubes were removed. The digest was acidified with 10 ml. of 5N HCl and the thiosulphate produced during the process of oxidation was titrated with $N/100$ $KIO_3.HIO_3$. A most satisfactory end-point was obtained by adding a slight excess of the biiodate and titrating back with thiosulphate. The methionine was calculated from the iodine required, 1 ml. of $N/100$ $KIO_3.HIO_3$ being equivalent to 0·1407 mg. of methionine-N or 1·49 mg. of methionine.

The methionine was also determined from the volatile iodide absorbed in the fourth and fifth absorbers. The contents of these were rinsed into a 100 ml. volumetric flask containing 25 ml. of 25 % sodium acetate solution. A little formic acid (about 10–12 drops) was added to reduce the bromine, the flask was shaken and the contents made up to the mark. Aliquot portions of 20 or 25 ml. were pipetted out, a little KI and a few drops of 10 % HCl were added and the liberated iodine was titrated with $N/100$ $Na_2S_2O_3$.

Sulphate. The H_2S and SO_2, produced by the reduction of sulphate, have been absorbed in the second absorber as CdS and $BaSO_3$, which may be quantitatively oxidized. The contents of the tube were rinsed into a beaker, a small excess of $N/100$ $KIO_3.HIO_3$ was added followed by a few crystals of KI and 2 ml. of 10 % HCl. After standing for a short time, the excess iodine was titrated with $N/100$ $Na_2S_2O_3$, 1 ml. of the iodate being equivalent to 0·16 mg. of sulphur.

Tests of the method. It was found advisable to keep both the condenser and the first three absorbers warm in order to ensure that the methyl iodide passed to the fourth and fifth absorbers. Lugg [1938, 1] recommended that the digestion of protein be commenced with the addition of 0·02 g. of KH_2PO_2 and that further quantities be added at intervals as long as iodine coloration was obvious. In hydrolysing impure leaf protein, however, the digest is dark orange in colour for reasons other than the presence of iodine, and it was found that the addition of 0·02 g. of KH_2PO_2 at the beginning and end of the hydrolysis was sufficient. Blank determinations were made with the HI at frequent intervals and corresponded to about 0·02–0·04 mg. of cystine-N, 0·03–0·04 mg. of S, 0·01 mg. of methionine-N by volatile iodide and 0·06–0·1 mg. of methionine-N by homocysteine.

In the examination of the method with a sample of edestin, containing 15·58 % N, with and without the phosphorus absorber, and with both cold and warm conditions for the condenser and the first three absorbers, there was not much difference between the figures either for cystine or for methionine by homocysteine, but the percentage of methionine by volatile iodide depended upon the conditions employed. The volatile iodide appeared to be detained when the conditions were cold and it seemed to be advisable to introduce the absorber containing red phosphorus. The percentage distribution of the sulphur in the edestin was found to correspond to 1·09 % cystine-N and 1·24 % methionine-N as compared with figures, obtained by other workers, of 0·89–1·31 for cystine-N and of 1·08–1·35 for methionine-N.

A number of preliminary trials were made of the recovery of cystine and methionine alone or mixed or added to edestin, the analyses being carried out under the conditions already described. The recovery of cystine averaged 97·5 % when examined alone, 99·3 % in presence of methionine and 98·8 % in presence of methionine and edestin. The recovery of methionine examined alone averaged 95·5 % by the volatile iodide method and 91·1 % by the homocysteine method, and the recovery was not affected by the presence of cystine or cystine and edestin.

With regard to the recovery of pure substances, Baernstein [1936] found values of 101·5 % for cystine and 99·4 and 99·6 % for methionine by the volatile iodide and homocysteine methods respectively. In the present investigation the recovery of cystine was satisfactory but the recovery of methionine was low. This, according to Kassell & Brand [1938], is probably due to the formation of methyl mercaptan. It is noteworthy that a small quantity of cystine, amounting on an average to 5·2 % of the methionine-N, was found in the estimation of methionine, suggesting that a small proportion of the homocysteine was titrated as cystine. When the analysis of a mixture of cystine and methionine was carried out, the recovery of cystine was therefore higher than when cystine was examined alone, while the recovery of methionine was not affected by the presence of cystine. The percentage recoveries were in good agreement with those found by Kassell & Brand [1938], who obtained 97·8 % for cystine and 93·7 and 89·3 % for methionine by the volatile iodide and homocysteine methods respectively.

Since the grass proteins contain both cystine and methionine, the correction factors used in the subsequent analysis were 1·007 for cystine and 1·047 or 1·098 for methionine according to the method of estimation. In the examination of the grass proteins, however, the yield of methionine was always about 25 % higher by the methyl iodide method than by the homocysteine method even with the above correction factors. It must be assumed that the protein preparations were not entirely free from impurities which could yield volatile iodide or could render methionine-sulphur insoluble.

Results

General. The results are all reported (Table 3) on a nitrogen basis, but do not include the figures for ten samples collected from manurial experiments. The distribution of S (Table 4) was calculated on the assumption that cystine and methionine were the only two S-containing amino-acids in the proteins and that the remainder of the S was in the form of sulphate. The figures for the sulphate-S were not corrected for the small amounts of H_2S liberated from cystine and methionine and corresponding to 1·25 and 0·89 % respectively of the cystine-S and methionine-S. The figures for methionine by the homocysteine method are given in Table 4.

The four species do not reveal noteworthy differences in their contents of cystine and methionine. The former varies from 0·90 to 1·40 % for white clover, 0·91 to 1·46 % for cocksfoot, 0·96 to 1·26 % for rye-grass and 0·82 to 1·32 % for timothy. The corresponding figures for methionine-N by homocysteine are 0·70 to 1·20 %, 0·73 to 1·24 %, 0·91 to 1·10 % and 0·74 to 1·29 %. In an examination of ten samples of leaf proteins by the same method, Lugg [1938, 1] found that the cystine varied from 1·1 to 1·5 % and the methionine from 1·2 to 1·6 % and that rather higher values were obtained by his differential oxidation method. Our figures are, therefore, somewhat lower than those obtained by Lugg. It will be observed that the legume, white clover, contained about the same amounts of the amino-acids as the grasses.

Table 3. *Cystine and methionine in grass protein preparations*

Nature of sample	% of total N in protein preparation as cystine- and/or cysteine-N				% of total N in protein preparation as methionine-N							
					By volatile iodide				By homocysteine			
	W.C.	C.	R.	T.	W.C.	C.	R.	T.	W.C.	C.	R.	T.
Young	1·18	1·28	1·04	0·86	1·22	1·59	1·27	1·04	1·04	1·17	1·04	0·78
	1·14		—	—	1·26		—	—	1·02	-	—	—
	1·03	—	-	—	1·30	—	—	-	0·94	—	—	—
Flowering or heading	1·33	1·37	1·16	1·13	1·40	1·52	1·30	1·17	1·15	1·12	1·10	0·91
	1·32	0·91	1·16	1·32	1·50	1·29	1·50	1·59	1·20	0·98	1·06	1·29
	1·40	1·32	1·26	—	1·46	1·52	1·43	—	1·14	1·20	1·10	—
	—	1·25	1·16	—	—	1·30	1·43	—	—	0·84	0·91	—
Old	1·35	1·46	1·12	1·24	1·38	1·30	1·52	1·53	1·13	1·22	1·01	1·19
	1·20	1·42	1·21	—	1·42	1·46	1·55	—	1·11	1·24	1·10	—
	1·36	1·42	—	—	1·48	1·54	—	—	1·20	1·21	—	-
Aftermath	1·01	1·01	0·96	1·20	1·20	1·21	1·40	1·38	0·90	0·73	1·05	1·12
	1·10	1·25	1·03	0·82	1·23	1·40	1·28	1·34	0·98	1·08	0·97	0·74
	—	1·05	0·98	—	—	1·41	1·34	-	-	0·84	0·89	—
	—	1·34	1·08	—		1·28	1·14	—	-	1·07	0·95	—
	—	1·16	1·03	-	—	1·50	1·20	—		1·09	0·94	—
		1·44	—	—	—	1·46	—	—	—	1·15	—	—
Grazed	0·90	1·13	1·10	0·85	1·29	1·35	1·18	1·40	0·70	1·03	1·00	0·97
	1·04	1·12	1·15	1·25	1·32	1·30	1·25	1·26	1·00	1·03	1·05	1·06
	—	-	1·22	-	—	—	1·08	-	—		0·95	—
		-	1·02	—	—	—	1·22	-	-	—	0·94	—
Average	1·18	1·25	1·11	1·08	1·34	1·40	1·31	1·34	1·04	1·06	1·00	1·01

Stage of growth. Lugg [1938, 2] could not find any association between the composition of extracted protein and the age of the leaves, but the results of this investigation show that stage of growth does affect both cystine and methionine. In general, the young and grazed samples contain less cystine and methionine than the older samples, the maximum figures being reached at about the stage of flowering or heading. This holds good for all four species with the single exception that the cystine-N in a sample of cocksfoot at flowering stage taken in July is the lowest figure for all the cocksfoot samples.

When the actual dates of sampling were considered it was obvious that the figures for clover reached a maximum in July and then decreased, whereas with the grasses the maximum in July was followed by a decline in August or September and another rise in September or October. This was the case for both cystine and methionine and this difference in seasonal variations between the clover and grasses may be explained by the fact that the growing period of the former is somewhat restricted, whereas the grasses continue to grow throughout the season.

The numbers of samples in the various age groups are too small to permit many positive assertions, but the figures have been submitted to statistical analysis and, where the number of degrees of freedom permitted, the significance of the differences of means has been tested by the usual t-technique—t being taken at 2·5. The laboratory standard errors (amounting to about ±2·5% for cystine and ±3·5% for methionine by homocysteine) were duly considered and significant differences were found to exist in the following cases:

Cystine. White clover. Flowering v. young or aftermath or grazed; old v. aftermath or grazed.

Cocksfoot. Old v. aftermath or grazed.

Rye-grass. Aftermath v. flowering or old.

Timothy. No significant differences.

Methionine. White clover. Young v. flowering; aftermath v. flowering or old.

Cocksfoot. Old v. aftermath.

Rye-grass and timothy. No significant differences emerge.

There seems to be little doubt, therefore, that both cystine and methionine rise to significant maxima when the plants have reached or passed the heading stage and that they are present in smaller amounts in the aftermath or grazed samples. This is supported by the results in Table 4, which show how the sulphur, other than that present in cystine or methionine, is almost invariably lower in the flowering or old samples than in the others. It is also of interest to note that there is a fairly strong relation between cystine and methionine, the latter on an average being about nine-tenths of the former.

Table 4. *Percentage distribution of S in protein preparations (methionine by homocysteine)*

Nature of sample	White clover			Cocksfoot			Rye-grass			Timothy		
	Cyst.	Meth.	Rest	Cyst.	Meth.	Rest	Cyst.	Meth.	Rest	Cyst.	Meth.	Rest
Young	43·4	38·3	18·3	41·9	38·2	19·9	36·3	36·3	27·4	38·8	35·2	26·0
	36·6	34·0	29·4	—	—	—	—	—	—	—	—	—
	41·1	37·4	21·5		—	—	—	—	—	—	—	—
Flowering or heading	47·9	41·4	10·7	45·4	37·2	17·4	47·1	43·0	9·9	49·7	40·0	10·4
	45·6	41·4	13·0	40·5	43·6	16·0	46·8	42·8	10·4	43·8	43·1	13·1
	47·6	38·8	13·7	43·3	39·4	17·4	47·7	41·7	10·6	—	—	—
	—	—	—	54·7	36·7	8·6	51·8	40·6	7·6	—	—	—
Old	48·9	40·9	10·1	48·9	40·9	10·2	47·7	43·0	9·3	46·7	44·8	8·6
	46·5	43·0	10·5	45·9	40·0	14·1	47·7	43·4	9·0	—	—	—
	46·6	41·1	12·3	47·2	40·3	12·5	—	—	—	—	—	—
Aftermath	37·3	33·3	29·4	45·9	33·1	21·0	39·4	43 1	17·6	43·4	40·5	16·1
	39·3	35·0	25 6	48·1	41·5	10·4	43·2	41·3	15·4	39·3	35·5	25·2
	—	—	—	44·4	35·6	20·0	40·5	36·8	22·8	—	—	—
	—		—	49·7	39·7	10·6	41·2	36·2	22·6	—	—	—
	—	—		42·5	39·9	17·6	44·1	40·2	15·7	—	—	—
	—	—	—	49·0	39·2	11·9	—	—	—	—	—	—
Grazed	40·7	31 6	27·7	41·4	37·8	20·8	43·1	39·1	17·8	37·4	42·6	20·0
	40·5	39·0	20·5	41·5	38·2	20·3	42·6	38·9	18·4	44·7	37·9	17·5
	—	—	—	—	—	—	50·9	39·6	9·5	—	—	—
	—	—	—	—		—	42·7	39·4	17·9	—	—	—
Average	43·2	38·1	18·7	45·6	38·8	15·5	44·6	40·3	15·1	43·0	40·0	16·9

There is an association between the total amino-N and the S-containing amino-acids in the proteins. The actual correlation coefficient for cystine + methionine and total amino-acids is +0·45, which is quite significant for sixty-three samples.

Environment. Although a substantial amount of evidence is available to show that the mineral matter in herbage is determined to a large degree by soil conditions, it is generally recognized that the composition of the organic matter is affected mainly by stage of growth. It was impossible to obtain samples at exactly the same stage of growth from the different sites in order to compare the influence of local soil and climatic conditions; from the data collected, however, any effects of locality were not apparent for any of the species concerned.

As has been pointed out elsewhere [Smith & Wang, 1941], grazing exerts a very important influence on the composition of grass, both the carotene and total N being considerably increased. This investigation shows that grass kept short by grazing contains cystine and methionine in amounts similar to those for young grass or aftermath. The effects of manuring were observed in only a few cases and were rather irregular and relatively unimportant. Lime produced a slight increase in the total amino-acids of the proteins of both cocksfoot and rye-grass, but the cystine and methionine were not altered to a significant extent. Slag had opposite effects on the two species. The effect of applying a dressing of ammonium sulphate at the rate of 1 cwt. per acre to rye-grass, 3 weeks before cutting, was to reduce the percentage of N as amino-N in the hydrolysed protein from 65 to 57 and to effect smaller reductions in the contents of cystine and methionine.

SUMMARY

Protein preparations have been made from sixty-three samples of *Trifolium repens*, *Dactylis glomerata*, *Lolium perenne* and *Phleum pratense*, by mincing and extraction with a buffer solution (*p*H 9·2), precipitation at *p*H 4·5, washing with alcohol and ether and finally drying in a vacuum.

The proteins contained 15–43 % of the total N in the plants. The amount of N in the protein was on the average about 11 %, being slightly higher in the preparations from the clover than in those from the grasses. The clover protein also contained a rather greater proportion of amino-N (67 %) after hydrolysis than the grass proteins (60–63 %).

The distribution of S as cystine, methionine and sulphate was estimated by hydrolysis with HI followed by determination of the cysteine and homocysteine in the digest and of methyl iodide, sulphide and sulphite in the absorption train. The values for methionine were much higher by the methyl iodide method than by the homocysteine method; expressed as percentage of N in the protein the averages were 1·35 and 1·03 % respectively. The amounts of cystine (1·16 %) and methionine were essentially the same for all four species but they were significantly greater in samples which had reached or passed flowering stage than in samples of young grass or aftermath or grass which had been kept short by grazing. The reverse was the case for sulphate-S which accounted for from about 8 to 29 % of the total S measured. Neither site nor manuring, with the exception of a late dressing of N, seemed to influence the results.

REFERENCES

Baernstein (1936). *J. biol. Chem.* **115**, 25, 33.
Chibnall (1939). Protein Metabolism in the Plant. Yale University Press.
Kassell & Brand (1938). *J. biol. Chem.* **125**, 145.
Levene & Van Slyke (1912). *J. biol. Chem.* **12**, 285.
Lugg (1938, 1). *Biochem. J.* **32**, 2114.
—— (1938, 2). *Biochem. J.* **32**, 2123.
—— (1939). *Biochem. J.* **33**, 110.
Smith & Wang (1941). *J. agric. Sci.* (in the Press).
Van Slyke (1911). *J. biol. Chem.* **9**, 185.
—— (1915). *J. biol. Chem.* **23**, 407.

41. THE PROTEINS OF *HEVEA BRASILIENSIS*
2. ANALYSIS OF A PRODUCT ISOLATED FROM CREPE RUBBER

By G. R. TRISTRAM

From the British Rubber Producers' Research Association, 48 Tewin Road,
Welwyn Garden City, Herts.

(*Received 28 February 1941*)

It has long been suspected that proteins played some part in the chemistry and technology of latex and raw rubber, but it is only recently that evidence has been obtained as to their exact function. It is now known [Bondy & Freundlich, 1938; Kemp & Straitiff, 1940] that the proteins in latex are the controlling factors in its stability. Thus, the coagulation point of latex corresponds to the isoelectric point of the proteins in solution; dialysed latex is not readily coagulated by acid unless a small concentration of salt is added, a property which is also common to the proteins. When latex is concentrated in the factory by centrifugal methods it is rendered much more unstable, presumably because it has lost a large amount of the protein which acted as a stabilizing agent.

Knowledge of the influence of proteins on the properties of raw rubber has been much more vague. It is known, however [Davis & Blake, 1937], that they do exert a considerable influence on the vulcanizing properties of rubber and that the water absorption of raw rubber depends on the amount of protein present [Skinner & Drakeley, 1931]. More recently Bloomfield & Farmer [1940] have shown that when crepe rubber is fractionated a series of N-free fractions are obtained which are soluble in the usual rubber solvents. The end-fraction which contains the bulk of the N is, however, insoluble. If, however, N-free rubber (prepared from trypsin-digested latex) is used as the starting material then all the fractions are soluble. Gee & Treloar [1940], using the fractions of Bloomfield & Farmer, have shown that the N-free fractions fall into a definite molecular series, whereas the fractions which contain N are radically different in behaviour.

Tristram [1940] described the isolation and analysis of a protein from dried fresh latex films. It was not known what relationship this product bore to the total protein of latex or to the protein which is associated with the hydrocarbon in raw rubber. De Vries [1924] expressed the opinion that latex contained two proteins, only one of which was precipitated with the rubber, but advanced no evidence to prove this view. Bondy & Freundlich [1938] isolated two protein fractions from ammonia-preserved latex and showed that both had isoelectric points within the precipitation range of latex but made no statement as to the presence of a third protein. Kemp & Straitiff [1940] repeating the work of Bondy & Freundlich claimed the presence of a third protein which was not coagulated with the hydrocarbon. Thus, if the findings of Kemp & Straitiff are correct, latex contains three proteins A, B and C, of which A and B are precipitated with the rubber. The preparation extracted from dried fresh latex films [Tristram, 1940] by borate may therefore be a mixture of A, B and C or, more probably, a sample rich in protein C. Unfortunately, none of the above workers characterized his preparation by analytical methods, so that it cannot be said definitely that A, B and C were separate entities. The present work was accordingly carried out to determine the relationship of this borate-extracted protein to the total protein of latex and to the protein found in raw rubber.

EXPERIMENTAL

The material chosen as the source of protein was the common commercial form, crepe rubber. The method of extraction first tried was that used previously [Tristram, 1940], but no protein was isolated. This suggests either that the protein in crepe rubber is somewhat denatured or that the protein is intimately associated with the rubber hydrocarbon. The latter seems to be more probable in view of the findings of Bloomfield & Farmer [1940] and of Gee & Treloar [1940].

The method of isolation adopted ultimately was based on that of Midgeley *et al.* [1937], since it depended on the dissolution of the rubber from the protein. The method has been modified in accordance with recent work in the above laboratories [Bloomfield & Farmer, 1940].

Method of isolation

500 g. shredded crepe rubber were extracted with acetone, in a drip extractor, for 24 hr. in an atmosphere of N_2. The residue was dried *in vacuo* and then extracted continuously with light petroleum under N_2 in an apparatus designed by Dr Michael [see Bloomfield & Farmer, 1940]. This procedure removed the '*sol*' rubber (*ca.* 50 % total), which was virtually nitrogen-free (0·05–0·02 %). The remainder, '*gel*' rubber, was coagulated with acetone, dried *in vacuo* and exhaustively extracted with xylene at 60–70°, during which time the mixture was disturbed as little as possible, the organic solvent being replaced by decantation every 24 hr. The residue was next refluxed *in vacuo* with xylene and the insoluble material removed in the centrifuge. The final residue, a gelatinous mass, was extracted successively with xylene, benzene and toluene and then dried *in vacuo*. The dry material was brittle and fibrous; it contained 34–35 % rubber, 8·13 % ash and 8·9 % N; the N content, ash- and rubber-free, was 14·6 %. So far it has not been found possible to separate the protein and rubber otherwise than by methods which involve breakdown of the protein, i.e. acid or alkaline hydrolysis. This finding is in keeping with that of Bloomfield & Farmer [1940] discussed above.

Analysis of product

The yield of protein, after correcting for the rubber content, was 2·3 g. or 0·46 % of the crepe. Owing to lack of material the analysis (Table 1, 2) could not be made so extensive as those reported previously (Table 1, 1), but it was

Table 1

(Results are given as percentage of total N)

	Dried latex 1	Crepe rubber 2
Method:	Borate buffer	Organic extraction
Yield g. %	0·1–0·15	0·46
Total N (ash- and rubber-free)	14·95	14·65
Amide-N	7·07	7·18
Tyrosine-N	3·3	3·1
Tryptophan-N	1·2	0·0–0·7
Arginine-N	12·9	13·0
Lysine-N	6·4	6·8
Glutamic acid-N	7·65	Not less than 1·8
Aspartic acid-N	6·5	Not less than 4·1

considered that values for ammonia, tyrosine, tryptophan, lysine and arginine would be sufficient for the purpose of comparison. In addition, the dicarboxylic acids have been investigated qualitatively to test the validity of the claim of Midgeley *et al.* [1937] that glutamic acid is absent from the protein of raw rubber.

DISCUSSION

The results show clearly that the two products have the same contents of ammonia, tyrosine, arginine and lysine, and in view of this agreement it is suggested that either there is only one protein in latex or that there is a mixture of proteins of which preparation 1 is a representative sample. The protein from crepe rubber was different in tryptophan content. One sample contained no tryptophan and a second, prepared from a new sample of crepe, contained 0·7 % of tryptophan-N. It is known that tryptophan is a labile amino-acid, being removed from the protein molecule extremely easily by digestion: it is more probable, however, that in the present case some change in the specificity of the amino-acid has occurred in the intact protein molecule during the manufacture of the crepe rubber from latex. ($NaHSO_3$ is often added to latex before coagulation with formic acid and after being coagulated and washed on the rollers the crepe is dried for 2 to 3 weeks in a tropical atmosphere (30° ±).) The loss of tryptophan is being investigated and the preliminary results indicate that $NaHSO_3$ does affect this amino-acid. Furthermore, proteinaceous material has been isolated from crepe rubber which had been oxidized in the presence of acetic acid [Bloomfield—unpublished experiments] and it has been found that this material contains only 0·2–0·3 % of tryptophan-N whereas the tyrosine-N is 2·8–3·1 %. These results suggest that the change in the specificity of tryptophan does take place during the manufacture of crepe rubber.

If the above suggestion is correct it follows that this protein or mixture of proteins is precipitated, more or less completely, on the coagulation of latex and is thus present in crepe rubber. Such a finding would be in keeping with the evidence of Bondy & Freundlich [1938] referred to above, but is in contrast to the ideas of De Vries [1924] and Kemp & Straitiff [1940]. It has been observed on numerous occasions [cf. Altman, 1939; Whitby & Greenberg (unpublished)] that some protein does, indeed, remain in the serum after coagulation of the latex. These workers, however, used serum prepared under factory conditions, and it is probable that some of the protein escaped precipitation since the *p*H would not be rigidly controlled. It is also possible that there are present in latex small amounts of proteinaceous material, probably a degradation product of the native proteins, which remains soluble at the coagulation point of the latex.

That the main bulk of the protein is precipitated is seen from the following experiment. A sample of latex preserved with sodium pentachlorophenate was found to contain 0·136 % protein-N. The dry rubber content was 38·0 %, so that the calculated N content of the rubber, assuming that the whole of the protein is precipitated, should be 0·37 %. A sample of the latex was then coagulated and the rubber creped under semi-factory conditions. The N content of the crepe was 0·37 %.

The analytical results given in Table 1 are not in complete agreement with the qualitative experiments of Midgeley *et al.* [1937], who obtained no evidence for the presence of cystine, tyrosine and glutamic acid in the proteins of raw rubber. The absence of tyrosine has also been suggested by Kemp & Straitiff [1940], who reported that the proteins of latex gave a negative Millon's test. It would appear that the analytical methods used by both sets of workers were inadequate for the purpose.

SUMMARY

A protein (2) *has been isolated from crepe rubber and analysed for amide-N*, tyrosine, tryptophan and the basic amino-acids. The dicarboxylic acids have been shown to be present.

The results indicate that preparation 2 is of the same composition as preparation 1 [Tristram, 1940].

It is now suggested that preparation 1 is a representative fraction of the total protein of latex.

Loss of tryptophan has been observed from the protein found in crepe rubber and a reason for this loss has been suggested.

The author wishes to thank Prof. A. C. Chibnall, who was responsible for directing the course of the research, and whose advice and criticism have proved invaluable.

The above work has been carried out as part of the programme of fundamental research on rubber undertaken by the Board of the British Rubber Producers' Research Association.

REFERENCES

Altman (1939). *Arch. Rubbercult.* **23**, 239.
Bloomfield & Farmer (1940). *I. R. I. Trans.* **16**, 69.
Bondy & Freundlich (1938). *C.R. Lab. Carlsberg*, **22**, 89.
Davis & Blake (1937). Chemistry and Technology of Rubber, p. 45. Reinhold.
De Vries (1924). *Arch. Rubbercult.* **8**, 264.
Gee & Treloar (1940). *I. R. I. Trans.* **16**, 184.
Kemp & Straitiff (1940). *J. phys. Chem.* **44**, 788.
Midgeley, Henne & Renoll (1937). *J. Amer. chem. Soc.* **59**, 2501.
Skinner & Drakeley (1931). *I. R. I. Trans.* **7**, 196.
Tristram (1940). *Biochem. J.* **34**, 301.

42. THE SEPARATION OF AMINO-ACIDS BY MEANS OF THEIR COPPER SALTS

3. THE HYDROLYSIS OF GLIADIN. AN INVESTIGATION OF THE DICARBOXYLATE FRACTION, INCLUDING THE ISOLATION OF *r*-GLUTAMIC ACID AS AN HYDROLYSIS PRODUCT

By BERNARD WILLIAM TOWN

From the Department of Chemistry, The Medical College,
St Bartholomew's Hospital

(*Received 30 January 1941*)

THE study of the hydrolysis of gliadin described in this paper was started during the tenure of an Alexander Brown Coxe Research Fellowship at Yale University during the year 1931–32. It is gradually being completed with a view to obtaining as complete a picture as possible of the disposition of the amino-acids in their separation by means of their copper salts.

In the original paper [Town, 1928] three fractions were obtained by separating the copper salts of the amino-acids into:

1. Those soluble in methyl alcohol.
2. Those soluble in water, but insoluble in methyl alcohol.
3. Those insoluble in water.

In a later paper [Town, 1936] the methyl alcohol-soluble copper salt fraction was further subdivided into (*a*) a part precipitated by alcoholic $CdCl_2$ and (*b*) the filtrate. The examination of the $CdCl_2$ precipitate, which contains the proline, was fully reported.

Modifications were introduced into this hydrolysis as a result of experience gained in previous hydrolyses. Important technical improvements were as follows.

(*a*) Copper hydroxide was used in place of the basic copper carbonate formerly employed. This substance is much more finely divided and more reactive, and when the amino-acids are evaporated with copper hydroxide to form their copper salts, the calculated quantity is sufficient, no excess being required.

(*b*) Earlier work had shown that unless the ammonia is first removed, cuprammonium compounds are formed during copper-salt formation and these interfere greatly with the true solubility of the copper salts of the amino-acids in the solvents employed, namely methyl alcohol and water.

(*c*) The dicarboxylates also interfered in the examination of the methyl alcohol-soluble copper salts [Town, 1936], for it was then found that 2·06 % of the glutamic acid, which should be present in the water-soluble copper salts, was isolated from the methyl alcohol-soluble copper salt fraction. This interfered with the isolation of proline.

The fact that both ammonia and the dicarboxylic amino-acids interfered during the copper salt separation suggested their prior removal. The dicarboxylates were first precipitated by the usual modification of Foreman's method

[Foreman, 1914], in which baryta takes the place of lime. The filtrate containing alcohol was then concentrated under reduced pressure to remove both the alcohol and the ammonia. The solution left was then freed from any excess baryta and converted into copper salts in the usual manner by evaporating with $Cu(OH)_2$.

The exhaustive examination of the amino-acids precipitated according to Foreman's method or by the common modification of using baryta in place of lime is of prime importance to protein chemists, for conflicting statements have been issued as to the extent that other amino-acids are precipitated as Ca or Ba salts insoluble in alcohol. The results of the present investigation do not seem to bear out any suggestion that other amino-acids are precipitated by the usual procedure. Thus 96·4 % of the nitrogen has been accounted for and the only amino-acids isolated from the dicarboxylate fraction were glutamic acid, aspartic acid and serine. The nitrogen distribution in the fraction is fully indicated in the scheme on p. 431.

The Ba salts of the dicarboxylic acids after precipitation were decomposed and the solution of the amino-acids, containing 27 % of the total N, concentrated and saturated with HCl gas. Five crops of glutamic acid hydrochloride were isolated, the first four crops being homogeneous and having M.P. above 202°. Details of their properties are given in Table 1. It will be noted that 87·5 % of the glutamic acid was isolated in the first crop. The first four crops yielded 254·5 g. of *l*(+)-glutamic acid hydrochloride in practically pure condition, whereas the 5th crop gave 3·0 g. of a fraction with a very low positive rotation and a low melting point. This was at first thought to be a very impure fraction, until it was found much later on to yield an anhydrous 3:5-dinitrobenzoyl derivative, M.P. 204° (51 % yield), whereas *l*(+)-glutamic acid gives rise to a similar derivative, M.P. 104° (40 % yield), crystallizing with one molecule of water.

A complete investigation of the mother liquor from the 5th crop increases the yield of this amino-acid with the 3:5-dinitrobenzoyl derivative, M.P. 204°, to 1·93 % of the dry protein or 1·06 % of the total N. A chemical investigation described below has shown it also to be a glutamic acid, and evidence is adduced which would lead to the belief that it is racemic glutamic acid (as distinct from the *dl* mixture), and that it is an hydrolysis product and not an artefact. The ratio of racemic to *l*(+)-glutamic acid is 1/19·6.

This isolation of racemized glutamic acid from a normal protein is of great interest in view of the discussion which has recently taken place between Kögl & Erxleben [1939, 1, 2 & 3] and Chibnall *et al*, [1939; 1940], concerning the glutamic acid of normal and malignant tissue proteins. The former authors asserted that the glutamic acid isolated from malignant tissue protein was partially racemized and they suggested that it was a characteristic of such proteins. Chibnall found that tumour proteins contained the bulk of their glutamic acid in the ordinary active form and he thought that any racemization was incidental to the methods employed in the isolation of the glutamic acid and had no special significance with respect to cancer tissue.

The isolation of racemic (as distinct from *dl*) glutamic acid from gliadin shows that a normal protein may contain some of its glutamic acid in the inactive racemic form and it may be a general characteristic of proteins. It would appear not to be a criterion of malignancy in living tissue. However, the suggestion that proteins may contain some portion of their constituent amino-acids in an optically inactive form is, as far as the author is aware, a new conception, and it will be interesting to see if in the future other amino-acids are shown to exist in the same state. The author contributed to a symposium which recently appeared on the subject of the glutamic acid of proteins [Town, 1940].

The total yield of glutamic acid isolated from the sample of gliadin used is 40·4 % of the dry protein or 22·1 % of the total N; these figures are made up as follows:

	% of dry protein		$\frac{\text{N isolated}}{\text{total N}}$	
l(+)-glutamic acid	37·81		20·7	
dl-(mixture)glutamic acid	0·69	2·62	0·38	1·44
r-(compound)glutamic acid	1·93		1·06	
	40·43		22·14	

It is interesting to note that the M.P. of *r*-glutamic acid hydrochloride is 194°, for divergent values for the M.P. of glutamic acid hydrochloride, are quoted in the literature. The following values for the M.P. of the different varieties of this substance have been found during the course of this work: *l*(+)-glutamic acid hydrochloride, 207°; *dl*-, 213°; *r*-, 194°.

The minute examination of the mother liquor from the precipitation of the glutamic acid hydrochloride was originally undertaken with a view to isolating hydroxyglutamic acid, which has been said to be present in gliadin. The isolation of this acid has proved difficult to many workers and although it was first isolated by Dakin as long ago as [1918] from caseinogen, its occurrence as an hydrolysis product of proteins cannot yet be said to have been substantiated. The mother liquors from the precipitation of glutamic acid hydrochloride were first re-precipitated with baryta and alcohol to ensure that only dicarboxylates were present, and then the free amino-acids were converted into copper salts to separate any aspartic acid as its insoluble copper salt. The soluble copper salts were freed from copper, and the free amino-acids treated with silver sulphate and baryta to precipitate any Ag hydroxyglutamate as recommended by Dakin [1918]. The treatment that the precipitate and filtrate from this Ag precipitation underwent is clearly indicated in Diagram 2 and it can be seen that no separation has been effected, for both precipitate and filtrate were found to contain *r*-glutamic acid. No trace of hydroxyglutamic acid was detected during the course of this work, although special care has been taken to look for it, and it is the author's considered opinion that it is not present in gliadin. It is interesting to note that 1·1 g. of serine have been isolated from the final fractions, and it seems probable that a mixture of say *r*-glutamic acid and serine may have been mistaken for this acid.

Another important point which this investigation has raised is the identity of copper aspartate. The copper aspartate isolated during this analysis appeared to be homogeneous, but on decomposition and fractional crystallization of the free amino-acid, it was shown to be a mixture of aspartic acid and *r*-glutamic acid in approximately equal proportions. Whether this is a general behaviour or something peculiar to this analysis has yet to be decided. The author has examined three laboratory samples of aspartic acid and all of them have been found to contain some proportion of the *r*-glutamic acid (the smallest proportion being approximately 4 %). Unfortunately their previous history is unknown. It might be mentioned that although aspartic acid is said to melt at 270°, it has been found in this laboratory that the only sample of aspartic acid which melted at all also contained *r*-glutamic acid and had M.P. 273° (indefinite). All pure samples of this acid prepared in this laboratory have M.P. >320°, and have not yet been made to melt.

EXPERIMENTAL

Preparation of the gliadin. A brief description is given owing to enquiries as to the method of preparation. 5 kg. of a commercial gluten preparation obtained from 'The Battle Creek Food Co.', Battle Creek, Michigan, were extracted with 25 l. and then with 10 l. of 73 % alcohol, each time being left overnight. The alcohol extract was poured off and the gluten residue wrapped in calico and squeezed in a hydraulic press. The extract was a clear yellow liquid (29 l.) and it was concentrated at 45° under reduced pressure to 1/3 volume. It was poured into ice-cold water and the gliadin precipitated as a thick viscous material which was transferred to a large basin and treated with three changes of dry acetone. The gliadin fell rapidly away to a fine powder and any lumps were passed with acetone through a hand mincing machine. The powder was left with dry acetone for several hours, filtered on a Büchner funnel, and spread on brown paper to dry.

Properties. The preparation is a fine white powder and dissolves in 73 % alcohol to a clear colourless solution. It contains 15·2 % N, 10·22 % moisture and 1·08 % fat (ether-soluble material). The N on a moisture and fat free basis is 17·40 %. The specific rotation using a 7 % solution of the air-dried specimen was −79·5°, which is equivalent to −88·8° for the anhydrous protein.

Hydrolysis

600 g. of the air-dry protein (532 g. anhydrous protein) were boiled under reflux for 36 hr. with 30 % w/v H_2SO_4. The total N in the hydrolysed protein was 91·6 g. The hydrolysed solution (3200 ml.) was diluted with 5100 ml. water and the humin material filtered off (8·6 g.; N, 0·3 g.). The filtrate was treated with hot aqueous baryta until just acid to Congo red, the $BaSO_4$ being filtered and washed with acidified boiling water (wt. $BaSO_4$, 2214 g.; N, 3·02 g.). The filtrate and washings were concentrated to 2870 ml. (N, 88·0 g.) and 786 g. of baryta in hot water added. To the resulting 5 l. of solution, 15 l. 95 % alcohol were added with constant stirring and the powdery but bulky precipitate of barium dicarboxylate allowed to settle overnight before filtration. The bulk of the supernatant alcohol, which was alkaline to litmus, was siphoned off and concentrated under reduced pressure (bath at 50°) to remove the ammonia before converting into copper salts. The Ba-dicarboxylate precipitate was finally filtered and washed once with 60 % alcohol.

The dicarboxylate precipitate. The precipitate of Ba salts was dissolved in dil. HCl (vol. solution 3 l.) and the Ba removed quantitatively; the $BaSO_4$ precipitate was washed twice by boiling with 800 ml. 5 % HCl (wt. $BaSO_4$, 740 g.; N, 0·86 g.). The amino-acid solution after concentration had volume 2300 ml. (N, 24·61 g.; 26·97 % of total N (91·62 g.)) and the concentration was continued until glutamic acid hydrochloride commenced to crystallize (vol. 1500 ml.). The solution was now cooled in ice and saturated with HCl gas; a large crystalline precipitate formed. It was filtered off, dissolved in 1200 ml. water and reprecipitated by saturation with HCl gas. The precipitate was filtered, and weighed 225 g. (1st crop).

The mother liquors were concentrated to 400 ml. and again saturated with HCl gas, giving a second crop of crystals which on recrystallization weighed 15 g. There was similarly obtained a 3rd crop (wt. 7·5 g.); the solution was now very dark brown and so it was boiled with norite (0·5 g.), filtered, concentrated to 100 ml. and again treated with HCl gas (4th crop, wt. 7·0 g.); on saturating the mother liquor (30 ml.) with HCl a 5th and final crop of hydrochloride was obtained (wt. 3·0 g.). The properties of these five crops of hydrochlorides are shown in Table 1, while the treatment of the mother liquor is described later.

Table 1

Crop	Wt. of hydrochloride g.	M.P. of hydrochloride °C.	Wt. as free glutamic acid g.	% N	% Cl	Test for NH_3	$[\alpha]_D$ ($c=4$ in H_2O)	Yield N/total N %	Yield Amino-acid/Protein %
1	225	207	181	7·55	19·40	—	+25·0	18·5	34·0
2	15	203	12·1	7·40	19·45	-	+20·8	1·1	2·3
3	7·5	202	6·0	7·43	19·5	—	+19·1	0·6	1·1
4	7·0	202	5·6	7·40	19·4	—	+24·0	0·6	1·1
5	3·0	175	2·4	7·45	19·3	Just + ve	+5·8	0·25	0·5
Total	257·5		207·1					21·05	39·0
							Excluding 5	20·8	38·5
Properties of glutamic acid hydrochloride:		Softens 203		7·63	19·34		+25·0		

Although the information in Table 1 was collected over a long time it has been put together without further explanation, although it should be pointed out that the rotations have only recently been determined.

The yield of glutamic acid expressed in terms of *l*(+)-, *d*(−)- and *dl*-mixture as calculated from the rotations is as follows:

% of protein

	l(+)-acid	*dl*(mixture)-acid	*l*(+)-acid	*d*(−)-acid
Crop 1	34·0	0·0	34·0	0·0
Crop 2	1·91	0·39	2·11	0·20
Crop 3	0·84	0·26	0·97	0·13
Crop 4	1·06	0·04	1·08	0·02
	37·81	0·69	38·16	0·35

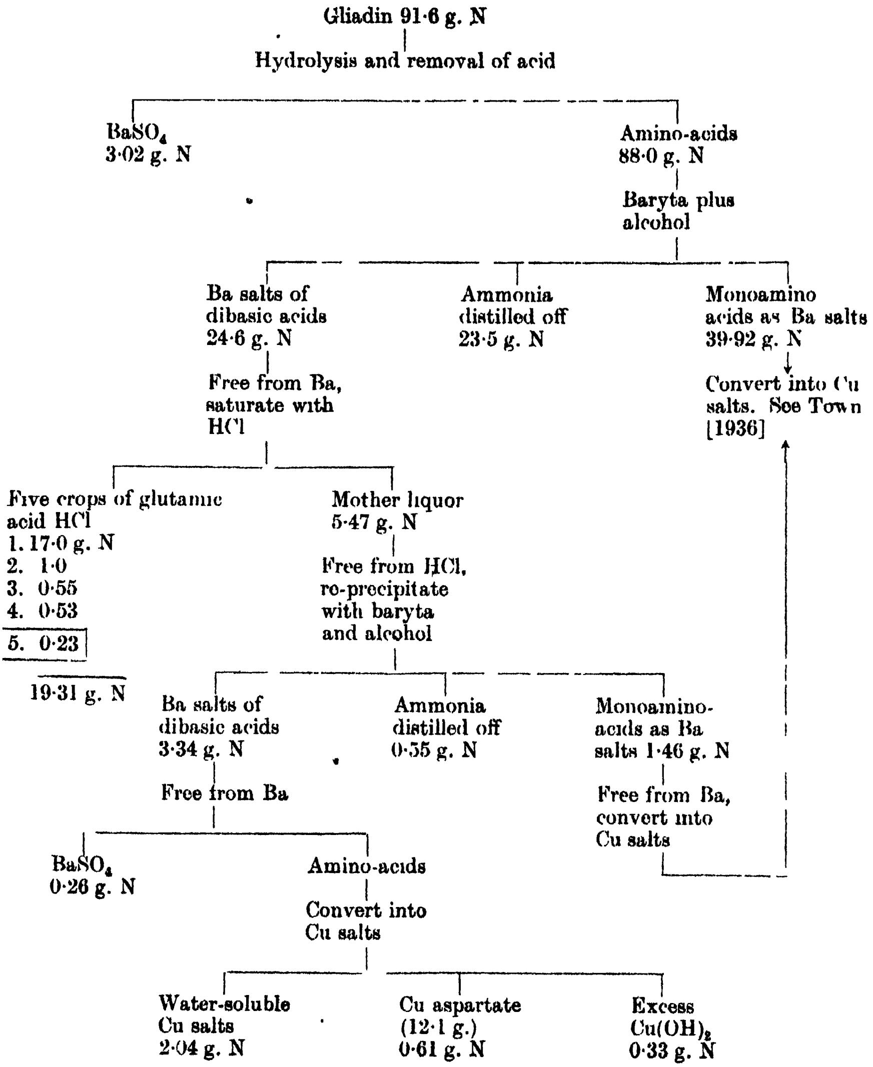
To this has to be added the amount of glutamic acid isolated from crop 5 and later fractions which is thought to be *r*-glutamic acid, namely 1·93 % of the dry protein. This makes a total yield of 40·4 %.

Examination of mother liquor from the precipitation of glutamic acid hydrochloride

The mother liquor from the 5th crop was evaporated to a syrup and then diluted to one litre; 25 ml. $12\,N$ H_2SO_4; were added and the HCl removed by adding Ag_2O to slight excess (test with baryta), excess Ag being removed with H_2S. The precipitates were washed by boiling twice with water (AgCl, wt. 58·6 g.; N, 0·01 g.: Ag_2S, wt. 1·3 g.: N, negligible); the filtrate and washings were concentrated to 710 ml. (N, 5·47 g.). It was decided at this stage to reprecipitate the dicarboxylates, if any, with the group reagent baryta and alcohol: 220 g. $Ba(OH)_2, 8H_2O$ were added and the combined volume was 800 ml.; 3 vol. 95 % alcohol were added and the granular precipitate allowed to settle, filtered and washed. Weight of precipitate 180 g. (N, 3·34 g.). The filtrate (3660 ml.; N, 2·02 g.) was evaporated under reduced pressure and the N in the aqueous residue determined (vol. 495 ml.; N, 1·46 g.). This indicates that 2·02–1·46, or 0·55 g., N was still present as ammonia and had distilled over. The distillate gave strongly positive tests for NH_3.

The aqueous residue (N, 1·46 g.) was converted into copper salts and mixed with the main bulk of copper salts of the monoamino-acids obtained after the original precipitation of the dicarboxylates. This action was later regretted, as it would have been interesting to see what amino-acids were precipitated with the dicarboxylates at their first precipitation, but one is in great danger of needlessly multiplying fractions. Before proceeding further, a schematic diagram will show the exact stage reached in the separation (Diagram 1).

Diagram 1



The precipitated Ba dicarboxylates (N, 3·34 g.) were dissolved in one litre of water and freed from Ba (wt. $BaSO_4$, 120 g.; N, 0·26 g.). The amino-acids were converted into copper salts by boiling with 25 g. $Cu(OH)_2$, filtered from excess $Cu(OH)_2$ (wt. 12 g.; N, 0·33 g. after boiling three times with water), and left in the ice-chest to cool; a mass of light blue feathery crystals of copper aspartate separated (wt. 12·1 g.; N, 0·61 g.; this represents 1·09 % of the dry protein or 0·66 % of total N; loss in weight on drying is 24·6 %; however, see later.)

The filtrate from the copper aspartate was freed from Cu (wt. CuS, 11 g.; N, 0·016 g.) and the solution concentrated to 1050 ml. (N, 2·04 g.). This solution was now treated as described by Dakin [1918] for the isolation of hydroxyglutamic acid. It was treated with precipitated AgOH and a little sulphuric acid (just sufficient to keep it acid to Congo red) until excess of Ag was present (brown precipitate with baryta). Baryta water was now added until *p*H 7 was reached and the white precipitate filtered off; on bringing the filtrate to *p*H 10 a small black precipitate formed which was also filtered off. The final filtrate had vol. 1430 ml. (N, 1·30 g.) and so the quantity of N precipitated should be 0·74 g., i.e. 36·3 % of the total. All fractions were freed from Ag (Ag_2S from precipitates 27·3 g.; N, 0·01 g.: Ag_2S from filtrate 13 g.; N, 0·006 g.) and the solutions concentrated on the water bath (65°) under reduced pressure.

Ag precipitate. On evaporation, tetrahedral crystals separated rather like glutamic acid, but the solution was evaporated to dryness and extracted by grinding with glacial acetic acid and left overnight. Only a small portion dissolved; the insoluble material being centrifuged off and again treated with acetic acid for 12 hr. Finally it was dissolved in 2 ml. H_2O and saturated with HCl gas (wt. of precipitate 1·7 g.). At first this was thought to be ordinary glutamic acid hydrochloride and was not examined. Later, however, on attempting to prepare the 3:5-dinitrobenzoyl derivative it was found to yield mainly a derivative, M.P. 204°, together with a trace of the normal glutamic acid derivative, M.P. 104°. Yield: 0·64 g. amino-acid hydrochloride gave 0·31 g. derivative, M.P. 204° (26·1 % of theory) and 0·02 g. derivative, M.P. 104° (1·5 %).

The material soluble in acetic acid was treated with methyl alcohol to cause granulation, dried and again treated with acetic acid. A trace (0·16 g.) was insoluble. The solution was concentrated to 30 ml. and poured into one litre of acetone when the material granulated: wt. 2·05 g.; N, 0·19 g.

Filtrate from Ag precipitation. As evaporation proceeded crystals separated in long needles which were identified as tyrosine (wt. 0·15 g.; N, 7·5 %; Millon positive); later a further crop of tetrahedral crystals separated (wt. 1·5 g., M.P. 197°), and finally a 3rd crop, also tetrahedra, containing a few coarse needles (wt. after crystallizing 0·8 g., M.P. 187–188°). Later it was found that both these fractions yielded 3:5-dinitrobenzoyl derivatives of M.P. 204° identical with the material obtained from the Ag precipitate. No further crops of crystals could be obtained and the syrupy residue was treated with glacial acetic acid, when all but 0·3 g. dissolved. The solution was evaporated *in vacuo* over NaOH and redissolved in glacial acetic acid, but 0·4 g. was insoluble. This 0·7 g. material was also found to yield a 3:5-dinitrobenzoyl derivative, M.P. 202°. The acetic acid solution was concentrated and granulated by pouring into one litre of acetone. The bulk of the material was insoluble (N 0·68 g.), but the acetone solution was slightly coloured; it was mixed with that from the Ag precipitate and was found to contain 0·24 g. N.

As the precipitation with silver and baryta according to the method of Dakin had apparently effected no separation the fractions were recombined to give (*a*) material insoluble in acetone containing 0·68 + 0·19 or 0·87 g. N; (*b*) material soluble in acetone (0·24 g. N). On evaporation both solutions gave syrupy residues and, after many experiments, these were dissolved separately in 50 % methyl alcohol and precipitated by adding a 20 % solution of mercuric acetate in 50 % methyl alcohol.

Acetone-soluble. N precipitated 0·10 g. (41·5 %); this was not examined further as it is probably diketopiperazine material.

Acetone-insoluble. Volume 310 ml. N precipitated 0·66 g. (76 % of total).

The mercury precipitate was decomposed and the solution on evaporation gave a syrup which on stirring suddenly solidified to a waxy solid; this became powdery on treatment with methyl alcohol (wt. 3·58 g.; N, 11·1 %; amino-N 97·5 %, M.P. 165°, softens 140°, no charring). The equiv. wt. by titrating in alcohol solution was 104; it formed a blue copper salt which did not crystallize. A further batch of this crystalline material (wt. 1·4 g.; N, 11·0 %) was obtained by evaporation and treatment with MeOH.

As this material seemed to be a mixture it was decided to mix the fractions and reprecipitate them with mercuric acetate. The N content of mixed crops was 0·54 g., of which 0·46 g. (87 %) was precipitated. The Hg precipitate was decomposed and the amino-acid solution evaporated under reduced pressure. It was difficult to work with, but on adding methyl alcohol till the solution was turbid and keeping, a quantity of hard gritty crystals formed (wt. 0·51 g.; N, 9·73 %; M.P. 188°), crystallizing in rectangular prisms. This was also later found to yield a 3:5-dinitrobenzoyl derivative M.P. 202°. The mother liquor on evaporation and retreatment with methyl alcohol yielded more crystals, also gritty to the touch (wt. 0·46 g.; N, 9·65 %). Its 3:5-dinitrobenzoyl derivative had M.P. 202°.

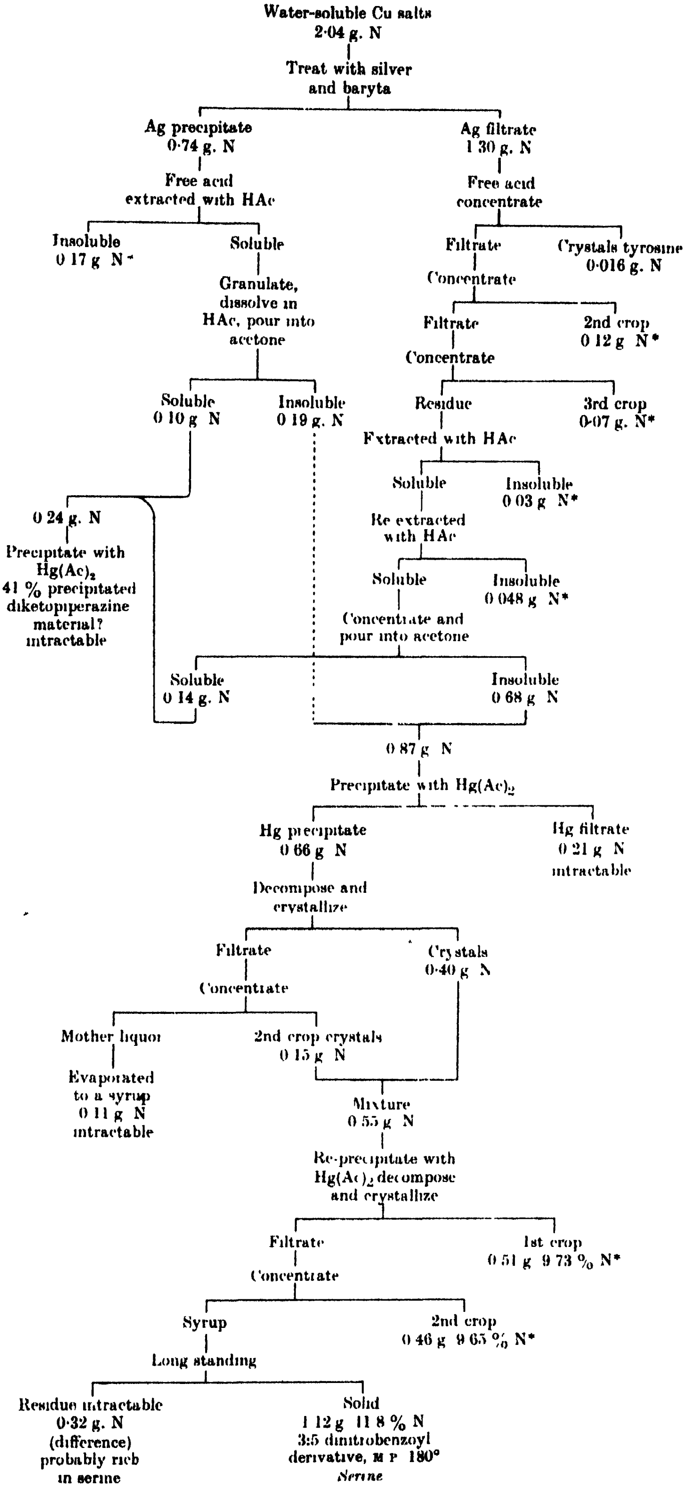
The residual syrup on long keeping became semi-solid, it was scraped out, filtered, dried for 3 weeks over conc. H_2SO_4 (wt. 1·12 g.); N, 11·8 %; moisture content 6·15 %; N in dry material 12·6 %). It was thought to be serine both from its N content and because it precipitated even in dilute solution with mercuric acetate in 50 % methyl alcohol, a property which the author has found to be characteristic of serine. It also yields a 3:5-dinitrobenzoyl derivative, M.P. 180°, mixed M.P. with a known *l*-serine derivative 181°, softens 179° (serine derivative, M.P. 183°).

The steps taken in the investigation of the water-soluble copper salts from the reprecipitation of the dicarboxylates, containing originally 2·04 g. N, are indicated schematically below (Diagram 2).

Examination of copper aspartate. This fraction which, from an examination of the feathery crystals of the copper salt, appeared to be homogeneous, was examined because the water content, 24·6 %, corresponded with 3·25 mol. of water of crystallization, whereas the figure quoted in the literature is 4·5 mol. The copper aspartate left, anhydrous wt. 7·12 g., was dissolved in 60 ml. *N* HCl, freed from copper and the solution evaporated and crystallized. By a complex process of crystallization from water and from alcohol, the free amino-acid was found to be a mixture. There was isolated 2·25 g. aspartic acid; M.P. >320°; C, 35·78; H, 5·00, N, 10·39 %: theory C, 36·08, H, 5·26, N, 10·53 %, and also 2·30 g. of an amino-acid, M.P. 194°, softening at 191°, which gave on analysis figures corresponding to glutamic acid; found, C, 40·2, H, 6·56, N, 9·76 %: theory C, 40·7, H, 6·90, N, 9·52 %: however, on treatment with 3:5-dinitrobenzoyl chloride it gave a derivative, M.P. 202°, softening at 200°, whereas natural glutamic acid yields a derivative M.P. 104°. As mentioned later in this paper this amino-acid is thought to be racemic glutamic acid, and what was thought to be copper aspartate is apparently a mixture or a double salt of copper aspartate and copper *r*-glutamate approximately in the ratio 1 : 1. This of course may be a phenomenon peculiar to this hydrolysis, but it is of great importance to ensure the purity of copper aspartate as aspartic acid is so frequently estimated as its copper salt.

Examination of the crops of glutamic acid hydrochloride. The details of the routine examination of these five crops are given in Table 1, and need no comment except to point out the low M.P. of crop 5 and to note its low rotation.

Diagram 2



All the fractions marked with an asterisk were found to give a 3:5-dinitrobenzoyl derivative M.P. 204°. This is the material which is considered to be *r*-glutamic acid. See p. 426 for full description.

Attempts were made to prepare the *p*-nitrophenylosazone of malic semialdehyde according to the method of Dakin [1918] from all these five crops in order to see if there was any β-hydroxyglutamic acid in the fractions, for its hydrochloride is known to be insoluble in HCl: 0·15 g. of each crop was used in the experiment and in each case crops 1, 2, 3 and 4, yielded no crystals from nitrobenzene. Crop 5 yielded 0·5 mg. of a dark red crystalline solid, M.P. 299° (uncorr.). This corresponds to a yield of 0·17 %, assuming crop 5 to be hydroxyglutamic acid for calculation purposes. The substance gave only the faintest blue colour with alcoholic NaOH, whereas *p*-nitrophenylosazones are said to give an intense blue colour with this reagent. These results were not very encouraging, and the fractions were put on one side.

Later [Town, 1941], it was found that glutamic acid yielded a well-characterized 3:5-dinitrobenzoyl derivative and so this derivative was prepared from each crop (see Table 1); 0·80 g. of material was used in each case equivalent to 1 g. acid chloride; only 0·4 g. of crop 5 was used.

	Wt. of deriv. g.	M.P. °	Yield %
Crop 1	0·62	104	40
Crop 2	0·62	104	40
Crop 3	0·61	104	39·4
Crop 4	0·59	104	38·1
Crop 5	0·40	203	51·6 calc. as glutamic acid deriv.

It can be seen from the M.P. and yield that crops 1–4 are undoubtedly homogeneous and consist of glutamic acid hydrochloride. Crop 5 is, however, quite different for it yields a derivative of much higher M.P. and also in a 25 % greater yield from only half the starting material. Careful attempts, not detailed here, have been made by fractionation to see if other substances were present, but no other 3:5-dinitrobenzoyl derivative than the one with M.P. 203° was isolated from this fraction.

After this, the same derivative was prepared from all the fractions which had been obtained in the attempt to isolate hydroxyglutamic acid from the residues from the precipitation of the glutamic acid as hydrochloride. It can be seen below that most of them consisted almost completely of the amino-acid yielding the 3:5-dinitrobenzoyl derivative with M.P. 203°. A similar amino-acid was also isolated by fractional crystallization of the aspartic acid obtained from the copper aspartate.

Examination of the amino-acid yielding a 3:5-dinitrobenzoyl derivative with M.P. *203–204°*

It is first of all important to study the disposition of this acid in the scheme of separation employed.

It occurs in crop 5 of the glutamic acid separation (Table 1); therefore its hydrochloride is sparingly soluble in strong HCl. It is present in the copper aspartate fraction, and therefore it either forms an insoluble double salt with copper aspartate or else its copper salt is itself sparingly soluble. It occurs both in the silver precipitate and filtrate on treatment with silver and baryta, but it is present to a greater extent in the filtrate; precipitation is thus only partial. The amino-acid is also partially soluble in glacial acetic acid.

The weights of material obtained from the various sources (see diagram 2) and also the yields of the 3:5-dinitrobenzoyl derivative obtained from the various fractions in which it occurs are indicated below. It is interesting to note that the yield of derivative is usually of the order 50–60 %, compared with one of 40 % in the case of *l*(+)-glutamic acid (see p. 426).

	Free amino-acid g.		Yield of deriv. %	
As hydrochloride 3·0 g. (crop 5)	2·4		51·6	
From Ag precipitate (diagram 2)	1·7		26	
From Ag filtrate—2nd crop	1·5		24	
From Ag filtrate (Table 3)—3rd crop	1·1		69	
0·79 g. insol. in glac. HAc	0·8		62	
0·51 g. from precipitation with $HgAc_2$	0·5		58	
0·46 g. with $HgAc_2$	0·46	impure	17	neglected
	8·0			

Also the copper aspartate (q.v.) yielded 2·3 g. of the same amino-acid giving a derivative melting at 202°. There have thus been isolated 10·3 g. of the material corresponding to a yield of 1·93 % of the dry protein or 1·07 % of the total N.

The hydrochloride of the amino-acid. Crop 5 of the hydrochlorides (Table 1) was dissolved in 17 ml. H_2O and saturated with HCl gas; on cooling and rubbing, it crystallized in fine needles, M.P. 194°, wt. 0·815 g. (wt. of starting material 1·570 g.). The mother liquor on evaporation to $\frac{1}{4}$ bulk was brown in colour and on cooling yielded a second fraction, wt. 0·29 g.; M.P. 194°. The two fractions were mixed and recrystallized and the derivative still had M.P. 194°. (Found: C, 32·87; H, 5·30; N, 7·53; Cl, 19·26 %. $C_5H_9O_4N.HCl$ requires C, 32·7; H, 5·45; N, 7·64; Cl, 19·35 %.) The amino-acid hydrochloride thus corresponds in composition with glutamic acid hydrochloride. A M.P. of 194° is among those quoted in the literature for glutamic acid hydrochloride, but it was not then appreciated that it could be the racemic variety.

The 3:5-dinitrobenzoyl derivative. This derivative was prepared in a similar manner to that of glutamic acid [Town, 1941]. It crystallizes in very thin hexagonal plates, M.P. 204°, which have a great tendency to overlay one another so that it is apt to crystallize in a large mass with only the ends of the crystals apparent. The crystal plates are so thin that good definition is difficult to obtain in photography. Photographs of the crystals have been obtained and copies can be sent to interested readers. The ordinary glutamic acid derivative, M.P. 104°, crystallizes in masses of very long fine needles. A 0·8 % solution in glacial acetic acid of the derivative melting at 204° gave no detectable rotation.

This derivative has no water of crystallization (the ordinary glutamic acid derivative crystallizes with 1 H_2O). (Found: C, 42·99; H, 3·15; N, 12·10 %; a substance of formula $C_{12}H_{11}N_3O_9$ has C, 42·4; H, 3·23; N, 12·30 %; which would correspond with the derivative from an acid of the formula $C_5H_9O_4N$.)

The free amino-acid. The residues of the free amino-acid, after the preparation of the 3:5-dinitrobenzoyl derivatives from all of them, were mixed (wt. 1·633 g.) and crystallized from 10 ml. H_2O; crystals readily separated on cooling, wt. 1·334 g., M.P. 203·5° softening slightly at 200·5°. The mother liquor on evaporation to 3 ml. deposited more crystals; wt. 0·042 g., M.P. 202·5°. The crystals were tetrahedral in shape, very hard to the touch, and practically insoluble in cold water. (Found: C, 41·02; H, 6·06; N, 9·38 %. $C_5H_9O_4N$ requires C, 40·7; H, 6·13; N, 9·52 %.)

The free acid thus also corresponds in composition with glutamic acid.

It was also determined that the ratio of amino-N/total N was 1/1.

Racemization of known glutamic acid. It was suggested that the material might be *dl*-glutamic acid and so 15 g. pure *l*(+)-glutamic acid were racemized by boiling in *N* NaOH and the rotation determined at 4 hr. intervals. Racemization was complete after 130 hr. boiling. The solution was decolorized with animal charcoal and after evaporation was extracted three times with 80 % alcohol. This extract in aqueous solution was saturated with HCl gas, and there were obtained 4·2 g. of pure *dl*-glutamic acid hydrochloride, M.P. 213° (sharp).

This yielded a 3:5-dinitrobenzoyl derivative (49 % yield), M.P. 104°—this seems to be the point at which the material dissolves in its own water of crystallization. The anhydrous derivative has M.P. 182°, softening at 179°. (Found: 5·05, 5·10 % H_2O corresponding to 1 H_2O per mol., similar to the glutamic acid derivative.) Thus the amino-acid isolated is not *dl*-glutamic acid.

The 3:5-dinitrobenzoyl derivative was also prepared from a sample of *d*(–)-glutamic acid kindly supplied by Prof. A. C. Chibnall; 0·1 g. of the acid gave a 62 % yield of the derivative which had M.P. 105°, mixed M.P. with known 104° derivative, 104°, softened at 101°. Loss in weight at 110° 5·12 %.

The possibility also existed that the amino-acid isolated might be methionine, which with a formula $C_5H_{11}SO_2N$ would give very similar analytical figures, but tests for S by Na fusion were negative. Before it was considered that the acid might be *r*-glutamic acid, it was thought probable that it might be either methylaspartic acid or the lactone of hydroxyglutamic acid and a decision could only be made by oxidation experiments.

Oxidation of the 3:5-dinitrobenzoyl derivative. It was decided to use this derivative in order to protect the amino group. 169 mg. of the derivative were dissolved in 5 ml. glacial acetic acid and the boiling solution titrated with 33·2 ml. *N*/10 $KMnO_4$ over the space of 5 min., equivalent to 2 atoms oxygen per mol. of derivative. The MnO_2 was filtered off and the solution evaporated on the water bath; solid separated when the volume was 3 ml.; wt. 47·5 mg., M.P. 164°, softened 159°. This, on crystallizing from 5 ml. boiling water, gave 21·4 mg. derivative, M.P. 182·5°, softening at 182°, crystallizing in rosettes of fine needles. (Found: C, 40·95; H, 2·57; N, 12·43 %. $C_{11}H_9N_3O_9$ or $C_4H_6O_4N.C_7H_3N_2O_5$ would require C, 40·45; H, 2·75; N, 12·43 %.) This derivative was not natural aspartic acid 3:5-dinitrobenzoyl derivative, however, in spite of its similar M.P., because a mixed M.P. with the derivative (M.P. 184·4°) gave M.P. 110° softening at 90°. Work is continuing on its identity.

Titration of the acid with alcoholic NaOH. 60 mg. of the acid in 5 ml. H_2O and 50 ml. ethyl alcohol were titrated with *N*/10 NaOH using thymolphthalein as indicator. As a check 60 mg. ordinary glutamic acid were treated similarly. Both solutions were back-titrated with *N*/10 HCl using methyl red as indicator. In both cases the ratio of $COOH/NH_2$ groups was 1·88/1 indicating that the acid is a dibasic monoamino-acid. The mol. wt. of the acid is 147·8 (glutamic acid 147).

The silver salt. The solution from the above titration was treated with an exact equivalent of *N*/10 $AgNO_3$ to free from Cl^- (wt. AgCl 14·5 mg.; should be 67·5 mg. thus some AgCl is retained in solution). The solution was decolorized by boiling with 0·25 g. charcoal and evaporated to a syrup; 150 mg. $AgNO_3$ in 2 ml. H_2O were added and the yellowish-white silver salt filtered off; wt. 120·2 mg. (theory is 147 mg. silver salt). This silver salt went black within an hour or so, and contained 60·1 % Ag. It was dissolved (112·3 mg.) in 5 ml. *N*/10 HNO_3, filtered from a trace of black residue (3·5 mg.) and brought to *p*H 7–8 (bromothymol blue) by adding 4 ml. *N*/10 NaOH; a whitish precipitate formed (46·7 mg.) which contained 8·49 % H_2O and 58·5 % Ag in the anhydrous salt. This salt also blackened readily on exposure to light. An acid of mol. wt. 147 would give a salt with 59·8 % Ag.

The copper salt. 28·6 mg. of acid in 5 ml. H_2O were boiled with 24 mg. $Cu(OH)_2$ (theory 19·3 mg.) and filtered; the deep blue solution on evaporation gave a blue glass, readily soluble in water, and readily precipitated by adding an equal volume of methyl alcohol as a bulky light blue amorphous powder. % Cu, 19·7. Anhydrous copper glutamate contains 30·3 % Cu, if Cu/N is 1/1.

Electrometric titration. The author's thanks are due to Prof. C. R. Harington for kindly arranging for an electrometric titration to be carried out on the amino-acid, and assuming the mol. wt. to be 147, the following pK values were determined. The figures for glutamic and aspartic acids are given for comparison.

	Amino-acid	Glutamic acid	Aspartic acid
pK_1	2·25	2·19	2·09
pK_2	4·17	4·28	3·87
pK_3	9·44	9·66	9·82

The figures afford strong evidence that the acid isolated is identical with glutamic acid.

Reduction. Attempts were made at reduction of the amino-acid using hydrogen in the presence of Pd adsorbed on charcoal as a catalyst to eliminate all possibility of it being the lactone of hydroxyglutamic acid. No evidence of the absorption of hydrogen was observed.

Oxidation. As the amino-acid thus undoubtedly appears to be a glutamic acid, it was decided to oxidise it with chloramine-T or other source of hypochlorous acid to β-cyanopropionic acid and thence by hydrolysis to succinic acid. Free hypochlorous acid was prepared by distilling a solution of Cl_2 water after treating it with mercuric oxide. On the basis that 1 g.-mol. of glutamic acid requires 2 mol. of HOCl (i.e. 4 equiv.) for oxidation to cyanopropionic acid, the exact equivalent of HOCl was added. 14·7 mg. of acid in 10 ml. solution were treated with 1·31 ml. HOCl (0·307 N) at 40°. After 30 min. the solution still gave a blue colour with starch-iodide paper, which became negative after warming for 2 min. The solution was evaporated on a water bath; 5 ml. 5N HCl were added and the solution evaporated to dryness. On keeping crystals formed; these were extracted with ether and yielded 5·7 mg. succinic acid, M.P. 181°, mixed M.P. with authentic succinic acid, 182–184°. A control experiment with 14·7 mg. of $l(+)$-glutamic acid yielded 6 mg. of succinic acid, M.P. 182–184°.

p*H of aqueous solution of the acid.* The pH of $M/100$ solutions (14·7 mg. in 10 ml. solution) of known and new glutamic acids were determined electrometrically and found to be respectively 3·35 and 3·31. This also tends to confirm the suggestion that the unknown acid is a glutamic acid.

Hydrolysis of 3:5-dinitrobenzoyl derivative of the acid. 210 mg. of the derivative were heated in a sealed tube at 120° with 5 ml. conc. HCl for 1 hr.; after cooling, the 3:5-dinitrobenzoic acid was filtered off: wt. 92 mg., 70 % theory. The solution was evaporated to give the free amino-acid hydrochloride (wt. 133 mg.), and this was dissolved in 9 % HCl and made up to 3 ml. The rotation was determined and the concentration of amino-acid by Kjeldahl analysis. Concentration, 95·6 mg. per 3 ml. solution; rotation, $-0{\cdot}15°$; hence $[\alpha]_D = -2{\cdot}36°$. Some doubt is felt about this rotation as the solution was slightly turbid and in the 2 dm. tube used it was very difficult to read; however, the value $-0{\cdot}15°$ is the mean of 24 readings. The value may well have been zero when compared with the value obtained below with known glutamic acid.

A similar experiment was carried out on the 3:5-dinitrobenzoyl derivative of known $l(+)$-glutamic acid. 220 mg. hydrolysed yielded 114 mg. 3:5-dinitrobenzoic acid (78 % theory) and the amino-acid was treated as above. Concentration of solution 76·7 mg. per 3 ml.; rotation +0·64°; and $[\alpha]_D = +12{\cdot}5°$. If this specific rotation is accurate and this solution was also slightly turbid, then it would appear that in the benzoylation process the *l*(+)-glutamic acid was racemized to the extent of 60 %; i.e. 30 % of the material was converted into its opposite enantiomorph, which however existed as the *dl* mixture and not as the racemic compound, for the 3:5-dinitrobenzoyl derivative had M.P. 104°, whereas the new amino-acid isolated yielded a derivative M.P. 204° and this is thought to be the racemic compound.

Re-benzoylation of the amino-acids obtained above. After the rotations of the free amino-acid hydrochlorides had been determined, the HCl was removed by evaporation and the residues treated with 0·15 g. of 3:5-dinitrobenzoyl chloride and NaOH in the usual manner.

The amino-acid from the derivative which had M.P. 204° gave a product which, after benzene extraction and crystallization of the benzene-insoluble portion from water, yielded 50 mg. of the 3:5-dinitrobenzoyl derivative and this still had M.P. 204°. The yield was 42·6 % calculated on the 85 mg. of acid which were left for this purpose. This corresponds with yields of the order of 50 % which were obtained using 10 times the quantity of starting material and would appear to indicate that no change has been caused by the repeated benzoylation.

The amino-acid from the derivative with M.P. 104°, which is that obtained from *l*(+)-, *d*(−)- and *dl*-glutamic acids, yielded by this same treatment 8 mg. of M.P. 204° derivative (a yield of 4·5 %); a mixed M.P. with known 204° derivative gave M.P. 201°, shrinking at 198°. Nothing more could be crystallized from the mother liquor, although careful attempts were made to increase the yield.

This experiment confirms the fact that the amino-acid which yields a 3:5-dinitrobenzoyl derivative M.P. 204° is a glutamic acid and also that it is the racemic compound, for all the other possibilities have been tried and found to yield the 104° M.P. derivative.

Although the *l*(+)-glutamic acid does, after two benzoylations, yield small quantities of the derivative with M.P. 204°, it is important to note the small yield in this case, only 4·5 %, compared with a yield of 42·6 % in the case of the amino-acid which originally gave the derivative with M.P. 204°. It can thus be seen that slight conversion of ordinary glutamic acid into the racemic compound does take place after repeated benzoylation, but compared with the yields obtained in the course of this analysis (from 50 to 60 %) after only *one* benzoylation it is negligible.

All the known experimental facts can be reconciled if it is presumed that the original amino-acid giving a 3:5-dinitrobenzoyl derivative M.P. 204° was racemic glutamic acid and owing to the great difficulty experienced in inducing ordinary *l*(+)-glutamic acid and also known *dl*-glutamic acid to form such a derivative, it is suggested that the *r*-glutamic acid is a hydrolysis product and not an artefact. It is hoped, when opportunity offers, to carry out investigations to determine the equilibrium conditions between *dl*- and *r*-glutamic acids.

Conclusion

The dicarboxylate fraction of gliadin has been exhaustively examined and the partition of the nitrogen in the various fractions is shown in the following balance sheet. Corrections have not been made for the nitrogen used in Kjeldahl determinations, as the nitrogen otherwise unaccounted for is considerably greater.

Nitrogen distribution in the dicarboxylate fraction of gliadin

Isolated			Original N content
l(+)-glutamic acid	19·0 g.		24·6 g.
dl(mixture)-glutamic	0·35 g.		
r(compound)-glutamic	0·97 g.		
		20·32 g.	
Non-dicarboxylate N after reprecipitation of mother liquors with baryta and alcohol		1·46 g.	
Ammonia present		0·55 g.	
Aspartic acid (2·25 g.)		0·24 g.	
Serine		0·13 g.	
Tyrosine		0·01 g.	
		22·71 g.	
Losses			
On AgCl	0·01 g.		
On $BaSO_4$ (2nd precipitation)	0·26 g.		
On excess $Cu(OH)_2$	0·33 g.		
On CuS	0·03 g.		
On Ag_2S	0·02 g.		
Soluble in acetone	0·24 g.		
$Hg(Ac)_2$ filtrate	0·08 g.		
Loss in crystallization of aspartic acid	0·04 g.		
		1·01 g.	23·7(2)
Unaccounted for		0·89 g.	
		24·6(1) g.	

Thus 23·7 g. N out of a total of 24·6 g. are accounted for, or 96·4 %, although just over 4 % represents unavoidable losses on precipitates. The only amino-acid present besides glutamic and aspartic acids, except for 1·46 g. N which did not precipitate with baryta and alcohol on the second treatment with this reagent (and which was mixed with the main bulk of copper salts), is a small quantity of serine. This is a very soluble amino-acid and its presence no doubt had a very profound effect on the crystallization of the final fractions. The presence of serine always tends to prevent the crystallization of other amino-acids.

SUMMARY

An investigation into the dicarboxylate fraction of gliadin, obtained by precipitating the dicarboxylates from the hydrolysed protein by means of baryta and alcohol, has been completed.

The yield of glutamic acid amounts to 40·4(3) % of the dry protein, or to 20·7 % of the total N.

Besides the ordinary *l*(+)-glutamic acid, there was isolated a new variety of glutamic acid which is believed to be racemic glutamic acid. This is thought to be a definite hydrolysis product and not an artefact. The ratio of racemic to *l*(+)-glutamic acid is 1/20.

The aspartic acid was isolated as a well-crystallized copper salt, but this was found to be a mixture, and to approximate to a double salt of copper *r*-glutamate and copper aspartate. This result may be peculiar to this analysis, but it indicates the necessity of ensuring the purity of copper aspartate in this method of estimation.

The yield of pure aspartic acid was 0·43 % of the dry protein, or 0·26 % of the total N.

The only other amino-acid present was serine, which was isolated to the extent of 0·18 % of the dry protein, or 0·14 % of the total N. It was identified

by its ready precipitation with mercuric acetate in 50 % methyl alcohol, and by its 3:5-dinitrobenzoyl derivative. This amino-acid no doubt accounted for the intractability of the mother liquors from the glutamic acid.

There seems no ground for the belief that baryta and alcohol, at least after a second precipitation with this reagent, bring down considerable quantities of other amino-acids.

Although only just published, a large part of this work was carried out during the tenure of an Alexander Brown Coxe Research Fellowship at Yale University, Connecticut, U.S.A., during the year 1931–32. Publication was delayed owing to the number of fractions whose identity had not been established. The work was continued at St Bartholomew's Hospital Medical College.

REFERENCES

Chibnall, Rees, Tristram, Williams & Boyland (1939). *Nature, Lond.*, **144**, 71.
—— —— & Williams (1940). *Nature, Lond.*, **145**, 311.
—— —— —— & Boyland (1940). *Biochem. J.* **34**, 285.
Dakin (1918). *Biochem. J.* **12**, 290.
Foreman (1914). *Biochem. J.* **8**, 463.
Kogl & Erxleben (1939, 1). *Hoppe-Seyl Z.* **258**, 57.
—— —— (1939, 2). *Hoppe-Seyl. Z.* **261**, 154.
—— —— (1939, 3). *Nature, Lond.*, **144**, 111.
Town (1928). *Biochem. J.* **22**, 1083.
—— (1936). *Biochem. J.* **30**, 1837.
—— (1940). *Nature, Lond.*, **145**, 312.
—— (1941). In course of publication.

43. AN ANALYSIS OF BENCE-JONES PROTEIN

BY JOHN DEVINE[1]

From the Liverpool School of Tropical Medicine

(*Received 21 January 1941*)

BENCE-JONES protein, which is characterized by precipitation from solution at low temperatures (45–50°) and resolution of the coagulum on boiling, is excreted in the urine in about 70 % of the cases of multiple myelomatosis and occasionally also in allied diseases of the bone marrow and blood-forming organs. Of some seven hundred papers on myelomatosis which have appeared since Bence-Jones [1848] first noted the excretion of a peculiar substance in a case of 'mollities ossium', relatively few have dealt in detail with the chemistry of the protein. The purpose of the present communication is to give the results of an analysis of the amino-acid content of a sample of Bence-Jones protein obtained from a case of multiple myelomatosis which recently came under observation in the terminal stages, in the Tropical Ward of the Royal Infirmary, Liverpool; other features of the case will be discussed elsewhere.

The urine, which was collected daily under toluene for about 3 weeks immediately before the death of the patient, showed the typical reactions of Bence-Jones protein and was free from normal plasma proteins. After storage at 0° the different samples were filtered and mixed; part was reserved for isolation of the protein in soluble form, and the rest was coagulated for analysis.

EXPERIMENTAL

As the amount of material for amino-acid analysis was small, attention was confined to colorimetric and small scale isolation methods. No attempt was made to apply the general solubility method devised by Bergmann & Stein [1939]. All the colorimetric analyses were carried out by means of a Hilger 'Spekker' Absorptiometer. In using this instrument the drum reading obtained by comparing the coloured solution with the appropriate blank is referred to a calibration curve similarly prepared from standard solutions of the pure constituent being estimated. The light filter is so chosen as to give maximum sensitivity. Solutions for the 1 cm. cups should be made up to 10 ml. All the reagents used were of A.R. quality or were purified by standard procedures and were freshly made up in solution. Blank tests were carried out where necessary and all the procedures were submitted to preliminary test against standard protein preparations. Unless otherwise stated, the results obtained with Bence-Jones protein are the mean of two or more determinations and refer to the moisture- and ash-free material.

Preparation of Bence-Jones protein. The *p*H of the urine was adjusted with acetic acid to the point of maximum precipitation in the cold and the coagulation completed by heating to 60° for 30 min. Exhaustive washing of the protein by centrifuging was carried out by allowing it to stand 1–2 days in the wash liquor and repeating this several times afresh—first with very dilute acetic acid, then water, water at 60°, aqueous alcohol, absolute alcohol, and finally light petroleum twice overnight.[1] The latter was repeated by extracting twice for a day in a Soxhlet apparatus and the protein was then air-dried under cover and finally in a desiccator over H_2SO_4.

---

[1] Research Fellow, Medical Research Council.

Methods

Moisture. A sample was dried to constant weight at 108° (about 4 hr.) in a silica crucible.

Ash. The residue from the moisture determination was ashed over a Bunsen flame and then kept at 600° for 2 hr. in a furnace.

Total N. By micro-Kjeldahl analysis. In a repeat experiment, with fresh standard solutions and $CuSO_4$-K_2SO_4 mixture as catalyst, the results were identical with those originally obtained by H_2SO_4-H_3PO_4-$K_2S_2O_8$ digestion.

Amide-N. About 0·07 g. was hydrolysed in a 6 × 1 in. test tube by heating for 6 hr. with 5 ml. 2*N* HCl [cf. Lugg, 1938, 3]. Part of the HCl was removed by concentration *in vacuo* and after neutralizing to phenolphthalein and adding 0·15 g. K_2CO_3 the solution was rapidly aerated for 2 hr. at 30° [Denis, 1910] and the NH_3 determined in *N*/50 acid in the usual manner.

Humin-N. The hydrolysate prepared for the determination of the basic amino-acids was filtered through a Whatman No. 1 paper and the residue washed well with distilled water and analysed by the Kjeldahl procedure.

Total S. Wet ashing by the Benedict-Denis method is known to yield faulty results as compared with the standard Parr bomb method [Painter & Franke, 1936; Masters, 1939] or the peroxide fusion method [Bailey, 1937] of which use was made in the present case following the directions of Lugg [1938, 2]. The usual procedure for the removal of Group 3 metals was carried out before precipitating $BaSO_4$.

Total P. About 0·05 g. protein was digested with 1 ml. conc. H_2SO_4 with the dropwise addition of a little perhydrol (Merck). The digest was neutralized and brought to 7 ml. and analysed by the method of Berenblum & Chain [1938]. Filter No. 3 (orange).

Tyrosine and tryptophan

The colorimetric method of Folin & Marenzi [1929] was used as adapted from that of Folin & Ciocalteu [1927]. From the limited data available, this appears to give substantially the same results as the later modification suggested by Lugg [1937]. In three determinations with about 0·08 g. protein, the time of hydrolysis and other details were varied. Separation of the tryptophan as the mercury complex was allowed to proceed overnight at 0° in each case. The tyrosine solutions were read immediately on mixing with the reagents as turbidity developed within a few minutes. The 30 min. development of the tryptophan colour with the Folin reagent was carried out in a bath at 20° or at room temperature and saturated solutions of Na_2CO_3 made at 18° and 25° respectively were used; the corresponding calibration curves were identical. Filters: Tyrosine No. 7 (dark blue), tryptophan No. 1 (red).

Dr R. A. Morton and Mr T. W. Goodwin kindly carried out an independent analysis by a slight modification of Holiday's [1936] spectrophotometric method. They conclude from experiments with casein (unpublished) that in order to make the difference between the observed absorption in the ultraviolet region and that calculated from the best available chemical data fit in with the extrapolated curve showing general absorption beyond 310mμ, it is necessary to carry out a hydrolysis of the protein with Lugg's [1938, 1] alkaline stannite reagent instead of merely dissolving it in NaOH. Apart from this modification, a preliminary determination on Bence-Jones protein in NaOH showed that the interference due to pigment was so great as to make the corrected (Holiday) result valueless; only a trace of tryptophan was evident. The more reliable results obtained on the colourless Lugg hydrolysate were: tyrosine 8·5 %, tryptophan 2·3 %. The corresponding figures obtained colorimetrically were 8·8 % and 1·4 % respectively, so that while the results for tyrosine compare well, those for tryptophan show a wide and unexplained divergence.

---

[1] For determination of the S-distribution according to Baernstein [1936, 1] it is necessary to eliminate traces of alcohol and ether and avoid drying above 100°. Lugg [1938, 2] states, however, that oven-drying does not affect the results appreciably.

Sulphur distribution

The discussions by Lugg [1932; 1933; 1938, 2] and Bailey [1937] of the various procedures for differentiating the total S fraction of proteins and the difficulties attendant on the colorimetric estimation of cystine led to the adoption in the present instance of Baernstein's [1936, 1, 2] elegant and comprehensive method. By hydrolysis with conc. HI a study of the S-distribution can be completed on 0·5 g. protein or less, including the determination of methionine as well as of cystine by titration and the estimation of any volatile S (H_2S+SO_2) arising during hydrolysis from SO_4 groups, etc., by absorption in $CdCl_2$-$BaCl_2$ mixture. On this basis it is claimed that almost all the S of a number of proteins can be quantitatively accounted for. The scheme does not permit of discrimination between cystine and cysteine. Certain refinements in the method suggested by Kassell & Brand [1938] do not appear to be essential [Macara & Plimmer, 1940].

Two traps instead of one were used for the volatile S, so that the efficiency of absorption could be gauged. These consisted of 10 ml. tapering centrifuge tubes with ground glass connexions, each containing 3·5 ml. wash liquor. A trial of the procedure with casein gave satisfactory results; the volatile S (0·016 mg.) was only enough to produce a faint turbidity with $CaCl_2$-$BaCl_2$. Recoveries of the amino-acids from a known mixture gave 98 % for cystine and 90 % for methionine, in agreement with the values reported by Kassell & Brand [1938] and Lugg [1938, 2].

Application of the method to Bence-Jones protein immediately made it obvious that a relatively large proportion of the S was being volatilized. A heavy precipitate formed in the first trap as soon as boiling commenced and necessitated, at the end of the reaction, careful washing out of the inlet tube with iodate mixture to decompose the deposit of sulphide. One determination on 0·369 g. protein yielded the following results, including the correction for recovery: cystine (cysteine + cystine) 2·91 %, methionine 0·58 %, volatile S 1·56 mg. After correction of the latter value by subtracting 1 % of the methionine-S and 2 % of the cystine-S [Kassell & Brand, 1938] the figures account almost quantitatively for the total S content of the protein: (cystine + methionine)-S 69·7 %; volatile S 28·2 %; total S recovery 97·9 %.

The basic amino-acids

A. By isolation

Arginine and lysine. The small scale adaptation by Block [1934] of the classical Kossel & Kutscher [1900] method for the determination of the basic amino-acids has been further modified recently [Block, 1938; Tristram, 1939].

The procedure advocated by Tristram for the isolation of arginine and lysine has been followed in the present investigation, since it avoids the use of excess $AgNO_3$ (which is liable to interfere with the estimation of lysine), gives greater delicacy in the adjustment of pH values, and incorporates the finding of Ayre [1938] that ammonium phosphotungstate is not decomposed by dilute H_2SO_4 in the presence of amyl alcohol-ether. The removal of NH_3 by NaOH before the isolation of lysine as picrate is thereby eliminated.

All the standard operations of concentrations *in vacuo*, etc., were adhered to. In the final stages of both analyses the operations were determined as usual by the total N content (Kjeldahl) of the solutions.

Histidine. While the figures recorded by Tristram lend no support to Block's [1938] contention that no more than about 75 % of the histidine present is recoverable as diflavianate, this amino-acid is not easy to deal with on account of the relatively small quantities present in most proteins and the difficulty of isolating the derivative in a state of purity. For this reason Block [1937] has suggested the use of nitranilic acid to precipitate histidine. In a later paper [Block, 1940], the recovery of histidine both from pure solutions and from protein hydrolysates is stated to be quantitative, using the factor 0·403 for the conversion of the nitranilate to histidine. This was checked on histidine solutions as follows: about 0·14 g. histidine hydrochloride monohydrate dissolved in 15 ml. water was precipitated by the addition of 0·24 g. nitranilic acid in 15 ml. methyl alcohol. The derivative crystallized immediately. The solution was left overnight in the ice-chest, filtered on a sintered glass crucible and the residue was washed with methyl alcohol and ether and dried in the air and for a short time at 108°. Recoveries were 100 % from B.D.H. histidine and 98·2 % from a slightly impure sample prepared from ox blood.

In applying this method to Bence-Jones protein, the histidine-Ag fraction was put through the whole procedure in use for the precipitation of histidine as diflavianate, i.e. removal of cystine and other impurities by successive treatment with $HgSO_4$ and $CuCO_3$. This is preferable to the shortened method proposed by Block [1938] in which the solution, after removal of Ag by H_2S, is merely treated with charcoal before addition of nitranilic acid. From the final solution (50 ml.) four 1 ml. aliquots were removed for colorimetric estimation as described below. The remainder, which was thereby calculated to contain about 19 mg. histidine, was concentrated *in vacuo* to 3 ml. in a 50 ml. distilling flask and 50 mg. nitranilic acid, dissolved in 7 ml. methyl alcohol, were added at room temperature. Crystallization was completed overnight at 0° and the precipitate collected quantitatively, as described before.

B. *By colorimetric methods.*

Where the available material allows only one gravimetric determination of the basic amino-acids to be made, the relative purity of the final histidine and arginine fractions provides a useful opportunity for checking the results colorimetrically, and safeguarding against the possibility of subsequent loss of material.

Histidine. Methods based on diazotization [e.g. Koessler & Hanke, 1919] have found little application. The Knoop [1908] reaction, which has been utilized by Kapeller-Adler [1933] for the quantitative estimation of histidine in pregnancy urines, has been applied recently to protein hydrolysates. An examination of recent papers on this reaction by Armstrong & Walker [1932], Földes [1936], Conrad & Berg [1937], Woolley & Peterson [1937] and Racker [1940] indicates that the procedure described by Block [1938] incorporates the most satisfactory modifications for use in protein analysis. The solution is treated at room temperature for 10 min. with the bromine reagent, slight excess of which is then destroyed by addition of a drop of arsenite solution. 2 ml. of ammonia-ammonium carbonate solution are added and the mixture is heated in the boiling water bath for 5 min., and then kept at room temperature for a further 10 min. for the complete development of the colour. It soon became evident on analysis of the histidine-Ag fraction from edestin that reasonably consistent results could not be obtained. Unfortunately, there is no record of individual variations apart from that which is implied in Block's directions to develop both unknown and standard solutions in sets of six, and then to mix each set before comparing. Woolley & Peterson [1937] claim a 97 % recovery of histidine from a yeast hydrolysate, whereas Racker [1940], in controlled tests on urine, states that the experimental error is about 10 %.

In experiments on pure histidine solutions no improvement was effected by carrying out the second stage of the reaction, which is probably the source of the trouble, at 90–95° with the object of minimizing the spitting and uneven heating of the liquid consequent on the rapid evolution of NH_3. An apparent improvement in this respect was achieved by the use of thin-walled, wider bore tubes (6 × ¾ in.). In studying the effects of temperature and heating period on the intensity and stability of the colour, it was clearly not advisable to aim for conditions of maximum intensity, since this was still not attained after 22 min. heating at 65°, 75° or 100°. The results suggested that the original directions to heat in the boiling water bath for 5 min. are preferable in that the colour is stable or almost so for about 10 min. after taking the first reading, though not for an hour as stated by Kapeller-Adler [1933]. For longer periods at 100° the colour is more intense but fades with increasing rapidity. Colours which were stable for at least 20 min. were also attained by heating at 75° for 12–17 min., but such conditions are less suitable for routine analysis. Under all the other conditions tested the solutions were unstable.

In following Block's directions (5 min. heating in a boiling water bath, etc.) for the construction of a calibration curve with the minor modifications suggested by the above, the mean deviation in the absorptiometer readings (Filter Nos. 5+6) averaged over each set of five determinations was 3·3 %. Over the range investigated (0·4–1·4 mg. histidine) the mean values for each set fell exactly on a straight line. Treatment of the histidine-Ag fraction of Bence-Jones protein with charcoal [Block, 1938] after removal of Ag with H_2S was unsatisfactory. The clear colourless solution gave a white precipitate on adding bromine and finally a turbid brown-violet solution. After further purification of the mother liquor in the usual manner with $HgSO_4$ and $CuCO_3$ the colour developed satisfactorily. The spread of the results was much greater than before, however, the four absorptiometer readings being 0·294, 0·271, 0·278, 0·257.

Arginine. Further comments on the procedures of Weber [1930] and of Jorpes & Thorén [1932] for the colorimetric estimation of arginine by means of the Sakaguchi reaction have recently been made by Fisher & Wilhelmi [1937], Davenport *et al.* [1938] and Thomas *et al.* [1939], and the choice of certain empirical factors has been more carefully defined. The time assigned to the first stage of the reaction, between arginine and the alkaline α-naphthol, before the addition of hypobromite, varies from 2–3 min. [Weber, 1930] to 1 hr. [Jorpes & Thorén, 1932]. A constant 0·20 ml. standard hypobromite solution (followed after a certain interval by 1 ml. 40 % urea to destroy the excess) was used by Jorpes & Thorén [1932] in developing the colour, and has been found satisfactory by Fisher & Wilhelmi [1937] and Davenport *et al.* [1938]. Thomas *et al.* [1939], however, have utilized Weber's [1930] suggestion that the optimum hypobromite concentration should be found by trial; this modification, which is designed for use with the Sheard-Sanford Photelometer, eliminates the preliminary separation of arginine from certain other amino-acids, etc., which interfere with the colour development, and is stated to be directly applicable to protein hydrolysates. For each standard and unknown, the amount of hypobromite is varied until the light absorption remains constant for about 10 min., i.e. the fading of the red colour, which is stable for not more than 2 min., is exactly compensated by the increasing yellow decomposition colour, so that on extrapolating to zero time the reading may be taken to represent 100 % red colour. The safest and most convenient interval allowable between the addition of hypobromite and that of urea has been fixed by Davenport *et al.* [1938], in the light of their experience with the similar estimation of glycocyamine, as 15 sec., in conformity with the earlier recommendations of Jorpes & Thorén [1932]. This allows of more accurate working than the shorter period of 4 sec. used by Weber [1930].

Following the directions of Thomas *et al.* [1939], the amount of hypobromite required to give a satisfactory colour with an aliquot containing 0·12 mg. arginine from the arginine fraction of edestin was found to be 0·20 ml. This amount was optimal also for standard solutions containing 0·08–0·20 mg. arginine. It was difficult to determine with consistency the small range over which the hypobromite could safely be varied so as to give readings which remained constant. On the other hand, excellent agreement was obtained in duplicate determinations, provided that the solutions were read immediately after making up to volume. This point was brought out in the actual determination on the appropriate fraction from Bence-Jones protein. Four groups containing 10 equal aliquots were treated with 0·20, 0·25, 0·30 and 0·35 ml. hypobromite respectively. All the readings were constant within the limits of the experimental error, viz., 1 %.

The method finally adopted, using the solutions recommended by Thomas *et al.* [1939], is as follows. The reagent bottles which are kept in the ice-chest are placed in a bath at 0°. A suitable aliquot of the arginine-Ag fraction (not more than 5 ml.) containing about 0·05–0·20 mg. arginine is placed in a thin-walled 6 × ¾ in. test tube graduated at 10 ml., made up to 5 ml. with distilled water and cooled in a separate ice-bath for 30 min. 1 ml. NaOH and 1 ml. α-naphthol are added and the tube replaced in the bath for 5 min. with occasional shaking. 0·20 ml. standard hypobromite solution is added and after exactly 15 sec. vigorous shaking 1 ml. 40 % urea is added with further shaking. The solution is brought to 10 ml. with water and mixed. After warming the tube slightly in the hand to prevent subsequent condensation of moisture, the solution is transferred to the absorptiometer cell as quickly as possible so that the diaphragm can be adjusted within 60–70 sec. after the addition of hypobromite. In adding the reagents the hypobromite is sucked up several times to cool the pipette (straight 1 ml. graduated to the tip) and is then blown directly into the liquid; after this has been shaken the pipette is 'blown off' against the inside of the tube just above the liquid. This allows sufficient time for further mixing before adding the urea solution similarly, blowing this out once from an Ostwald pipette. Holders for the pipettes were not found necessary.

Filter combination Nos. 5 + 6 is the most sensitive.

Summary of analysis of the basic amino-acids in 2·490 g. Bence-Jones protein

With appropriate corrections for aliquots removed for N determination and colorimetric analysis and for the solubility of arginine flavianate and lysine picrate and the incidental losses of procedure [Tristram, 1939], the results are as follows:

		Wt. found (mg.)	Mean % of protein
Histidine:	By nitranilate	21·3	0·90
	Colorimetrically	23·6	
Arginine:	By flavianate	89·5	3·64
	Colorimetrically	91·5	
Lysine*:	By picrate	101·0	4·04

* The lysine picrate exploded at 256° and after recrystallization at 266°.

It may be noted that the minimum mol. wt. of the protein calculated from the histidine content (0·9 %) is 17,200; or assuming 2 mol. histidine per mol. protein, 34,400. This agrees well with the value of 35,000 determined by Svedberg & Sjögren [1929].

Phenylalanine

The colorimetric nitration method of Kapeller-Adler [1932] appears to be the only small scale method which has been proposed for the determination of phenylalanine. Jervis *et al.* [1940] have suggested, contrary to the original procedure described by Block [1938], that the removal of histidine and tyrosine may be omitted if the final solution is examined with a suitable filter. The full procedure is outlined here as conducted with slight modifications on one-fifth of the weight of protein suggested by Kapeller-Adler.

0·4 g. protein is refluxed for 20 hr. with 5 ml. 25 % H_2SO_4 in a micro-Kjeldahl flask. The humin is removed by centrifuging and washed 3 times with cold water. The solution and washings are brought to 40 ml. (filtered if necessary) and three 10 ml. aliquots are placed in 15 ml. centrifuge tubes and treated with 0·5 ml., 1·0 ml. and 1·5 ml. respectively of 10 % aqueous phosphotungstic acid. Each mixture is well stirred and the precipitate partially dissolved by warming the tube in a water bath. The solution is allowed to cool overnight, the precipitate washed with very dilute H_2SO_4 and the filtrate and washings in each case filtered through a small paper into a 50 ml. beaker, the outside of which is marked at the 10 and 20 ml. levels. The filtration ensures the elimination of phosphotungstate scum which is not easily centrifuged down. The solution is treated in the cold with $N/10$ $KMnO_4$ in small portions till the mixture is faintly coloured and then concentrated over a small flame. This operation is too tedious on the water bath. From time to time $KMnO_4$ solution is added to ensure that excess is present. When a volume of about 4 ml. is reached, further concentration to the oily stage (1–2 ml.) is effected very cautiously. 2 ml. of 10 % KNO_3 in conc. H_2SO_4 are added to the cooled mixture, the whole heated for 20 min. on the water bath and finally brought to about 6 ml. The beaker is next cooled in an ice bath and 5 ml. 15 % hydroxylamine hydrochloride added dropwise with mixing. After cooling to 0° again conc. NH_4OH is added slowly with mixing to a volume of about 20 ml. and the solution is transferred to a 25 ml. flask and brought to volume with conc. NH_4OH. The contents are thoroughly mixed and the development of the colour completed by placing the flask in a bath at 40° for 5 min. and then in an ice bath for a further 15 min. The three solutions thus prepared are read with the No. 6 filter (light blue). The determination is repeated if necessary using only the optimum amount of phosphotungstic acid. Occasionally a solution may develop a brownish shade and should be discarded. A slight deviation in the shade of the unknown from that of the standard solution which would make matching difficult in the visual type of colorimeter can be ignored. It was not found necessary to submit the protein hydrolysate to preliminary fractionation, as recently suggested by Macara & Plimmer [1940] who were unable to develop satisfactory colours with dephosphocaseose.

For the 1 cm. cell a suitable range of values for the calibration curve is 1–8 mg. phenylalanine.

Hydroxyproline

No small scale gravimetric method has yet been developed for the determination of hydroxyproline. The colorimetric method of Waldschmidt-Leitz & Akabori [1934] for the micro-estimation of both proline and hydroxyproline in the same sample, which is developed from that of Lang [1933], requires steam distillation in a specially designed apparatus and yields a value of only 8·9–9·4 % for the hydroxyproline content of gelatin as compared with the accepted value of 14·4 % [Bergmann, 1935]. The principle of oxidizing the hydroxyproline and coupling the product with isatin has been modified along simpler lines by McFarlane & Guest [1939] who use as the oxidizing agent alkaline hydrogen peroxide in the presence of a small amount of $CuSO_4$ as catalyst. This method is stated to yield a value of 14·65 %.

Using this procedure on a sample of grade B gelatin (*a*) 0·5 g. refluxed 25 hr. with 8 ml. 20 % HCl, (*b*) 0·5 g. autoclaved with 10 ml. 3*N* HCl for 6 hr. at 145°, lower but identical values in each case of 10·4 % were obtained. As the hydroxyproline contents of most proteins are much less than that of gelatin, the colours developed from more concentrated hydrolysates (with appropriate dilution after development) were compared with those prepared according to the original procedure, but were in each case found to be less intense: no improvement was effected by increasing the concentration of reagents. Hence, where possible the final dilution should be avoided, the colour being developed in a larger volume of solution to enable the 4 cm. cells to be employed in the absorptiometer (Filter No. 6).

Tests made on Bence-Jones protein ((*a*) 0·25 g. refluxed 24 hr. with 5 ml. 20 % HCl and the hydrolysate brought to 25 ml., (*b*) 0·1 g. autoclaved 6 hr. at 145° in 3*N* HCl and the hydrolysate brought to 10 ml.) showed no trace of colour as compared with a control which contained the same reagents but which had not been heated. As an obvious colour was developed from 2 ml. standard solution containing 0·08 mg. hydroxyproline (B.D.H.), which is the smallest amount that could have been present in the test aliquots, assuming only 1 mol. hydroxyproline per mol. protein (mol. wt. 35,000), it can safely be concluded that this amino-acid is completely absent from Bence-Jones protein. This was confirmed later on the filtrate from the proline estimation.

Proline

The method of Engeland & Bastian [1937], which involves methylation of the mixed amino-acids and the isolation of dimethylproline by successive precipitation with $HgCl_2$, H_2PtCl_6 and $HAuCl_4$, has been used by Bastian [1937] who records a figure of 25–26·5 % for the proline content of gelatin as compared with the accepted figure of 19·7 % [Bergmann, 1935]. Guest [1939] has recently made use of the well-known condensation between oxidized proline and *p*-dimethylaminobenzaldehyde in acid solution in a method in which the oxidation is effected by means of a suspension of PbO_2 in a phosphate buffer pH 8·7. As this procedure is stated to yield a satisfactory value for the proline content of casein and depends on there being little or no hydroxyproline present, a considerable number of tests and modifications, which need not be detailed, were made, but the method was finally abandoned. Consistent results could not be obtained with either pure proline or hydrolysates of casein or Bence-Jones protein. Moreover, in preliminary experiments with casein, the colours developed after acid hydrolysis (HCl and H_2SO_4) were so weak compared with those of baryta hydrolysates as to make the method suspect. The possibility was therefore examined of precipitating the proline on a small scale by means of ammonium rhodanilate [Bergmann, 1935].

In the large scale precipitation of proline, Bergmann has used various reagents including flavianic acid [Bergmann, 1935], phosphotungstic acid [Bergmann & Niemann, 1936] and alcohol [Bergmann & Niemann, 1937], mainly to remove arginine from the hydrolysate before addition of ammonium rhodanilate. Using aliquots from the same hydrolysate equivalent to 1·6–5·0 g. gelatin (Coignet Gold Label) the addition of flavianic acid was found to be unsatisfactory since it is difficult to remove excess from the precipitate of proline rhodanilate by washing on the filter; on the other hand, phosphotungstic acid results in a much cleaner solution and the amount added can be varied within fairly wide limits without affecting the result. As the final precipitate may easily be contaminated with excess of the ammonium rhodanilate which is not very soluble in water, it is necessary to have a fairly accurate idea of the amount of proline present, which may entail several trials, and to adhere to a fixed ratio between the volume of hydrolysate and that of the reagent. The correction factor for the solubility of proline rhodanilate as determined by Bergmann [1935] can then be applied. The details of the method finally adopted are as follows.

About 0·5 g. Bence-Jones protein was refluxed for 24 hr. with 10 ml. 20 % HCl. The humin was removed by centrifuging and washed twice with cold water. The total solution was concentrated three times *in vacuo* to remove HCl, transferred quantitatively to a 15 ml. centrifuge tube and treated dropwise with 1·0–1·4 ml. 10 % phosphotungstic acid, mixing well with a fine rod. After standing in the ice chest for 2 hr. the mixture was centrifuged and the liquid poured off and concentrated *in vacuo* to a gum. The residue was taken up in 3 ml. water and the solution transferred by means of a small teat pipette to a wide bore graduated test tube and adjusted to 4 ml. A solution of 0·14 g. ammonium rhodanilate in 2·4 ml. methyl alcohol was placed in the flask and the amino-acid solution pipetted in slowly with shaking, the pipette and test tube being washed with 2–3 drops methyl alcohol. After 2 hr. on ice the mixture was filtered on a sintered glass crucible and the residue washed three times with ice-cold water and dried in a desiccator. 0·460 g. protein gave 0·1345 g. proline rhodanilate (M.P. 133° as required). According to Bergmann's [1935] finding, the solubility correction[1] for 6·6 ml. mother liquor (37 % methyl alcohol) is 0·0165 g., giving a total of 0·151 g. proline rhodanilate or 0·19 × 0·151 g. proline. This is equivalent to 6·24 % of the moisture- and ash-free protein.

In one of the determinations the filtrate from the proline precipitation, without washings, was treated with 1 g. ammonium reineckate + 2 drops pyridine and left for 2 hr. on ice as described by Bergmann [1935] for the isolation of hydroxyproline. The mixture was then filtered and the residue washed well and decomposed on the filter in aqueous suspension by adding 2 ml. pyridine. The resulting filtrate was acidified with acetic acid, the precipitate removed by centrifuging and the solution then tested for hydroxyproline by Guest's [1939] method. No trace of colour was produced, thus confirming the previous results.

---

[1] Bergmann [1935] precipitated the proline rhodanilate from a solution containing 20 g. in 150 ml. methyl alcohol by adding 250 ml. 0·5 N HCl and recovered 19 g., i.e. a solubility of 1 g. in 400 ml. 37 % methyl alcohol which he loosely applies as a + 5 % correction.

SUMMARY AND DISCUSSION

The values for the various constituents are recorded in Table 1, along with those found by other investigators.

Table 1. *Comparison of analyses of Bence-Jones protein*

All the values are calculated as percentage of the moisture- and ash-free protein unless otherwise stated.

Constituent	Hopkins & Savory*	Abderhalden & Rostoski [1905]	Calvery & Freyberg†	Present author	Miscellaneous‡ 3	Miscellaneous‡ 4	Miscellaneous‡ 5
Ash	Trace	—	1·2–5·1	0·4	—	—	—
Moisture	Nil	—	4·8–10·6	7·5	—	—	—
Total N	16·2	—	18·1	14·7	14·8	16·07	16·01
Amino-N	—	—	14·3	—	—	—	—
Amide-N	1·3	—	1·0	1·0	—	—	—
Humin-N	—	—	Nil	0·14	—	—	—
Sulphur	1·18	—	1·0	1·34	—	—	1·24
Phosphorus	-	—	Nil	<0·01	<0·01	—	—
Tyrosine	4·20	1·7	6·7	8·8 (8·5)§	6·1	—	—
Tryptophan	0·82	—	2·5	1·4 (2·3)§	1·7	4·2	—
Cystine	0·57	—	3·0	2·9	—	—	—
Methionine	—	—	—	0·58	—	-	—
Arginine	6·04	—	5·1	3·6	—	—	—
Histidine	0·81	—	1·2	0·90	—	—	-
Lysine	3·67	—	6·8	4·0	-	—	
Glycine	Present	1·7	—	—	—	—	—
Alanine	Present?	4·5	—	—	—	—	—
Leucine	6·51	10·6	—	—	—	—	—
Valine	5·60	—	—	-	—	—	-
Aspartic acid	2·12	4·5	4·6	—	—	—	—
Glutamic acid	7·8	6·0	8·6	—	—	—	—
Proline	2·70	1·9	—	6·3	—	—	-
Hydroxyproline	—	—	—	Nil	—	—	—
Phenylalanine	4·83	1·5	—	6·1	—	—	—

* The values of Hopkins & Savory [1911] are the mean of two consistent sets obtained from the protein of two separate patients.

† The values of Calvery & Freyberg [1935] are the mean for two specimens of protein excreted at different times by the same patient.

‡ 3, Hewitt [1929]; values uncorrected for ash. 4, Ohlsson & Nordh [1929]. 5, Magnus-Levy [1936].

§ Spectroscopic analysis.

It is of interest to consider these results firstly in relation to other evidence bearing on the nature of Bence-Jones protein. Physicochemical properties of the protein, including the refractive index, optical rotatory power and ultraviolet absorption spectrum have been investigated by Hewitt [1929]. The isoelectric point of different samples has been determined cataphoretically [Svedberg & Sjögren, 1929; Mainzer, 1932, 1, 2, 3; McFarlane, 1935; Magnus-Levy, 1936; Kekwick, 1940] as well as by electrometric titration [Jervell, 1932] and flocculation [cf. Magnus-Levy, 1936]. Svedberg & Sjögren [1929] and Magnus-Levy [1936] have reported measurements of the molecular weight by means of the ultracentrifuge and further work along these lines has been reported by Kekwick [1940] and others. The results of these investigations confirm the immunological findings of Bayne-Jones & Wilson [1922, 1, 2], Robinson [1927] and others that Bence-Jones protein is of variable composition and is distinct from normal plasma proteins.

The extension of this work to the determination of the chemical structure of the protein has received little attention. Apart from the investigations of Lüscher [1922], Medes *et al.* [1935] and Tsutsui [1935] on the N-distribution, amino-acid analyses have been reported in only three cases [Abderhalden & Rostoski, 1905; Hopkins & Savory, 1911; Calvery & Freyberg, 1935]. While there are not as yet sufficient trustworthy data on the composition of the protein to give any certain indication of its pathological origin, there are however several points of interest in comparing the various values. Medes *et al.* [1935] examined, by the Van Slyke method, six different samples of the protein. Five of these were shown to be chemically identical within the limits of the method, the analyses being in close agreement with that of Luscher [1922], while the sixth was quite different. Lüscher's [1922] analysis was made on material originally examined by Hopkins & Savory [1911] who considered that despite the admittedly imperfect methods of isolation then available, both the proteins examined by them as obtained from different patients were identical but distinct from the sample analysed by Abderhalden & Rostoski [1905]. Comparison of the figures obtained more recently by Calvery & Freyberg [1935] with those of the present paper and with miscellaneous values shows several divergencies. My N value is low, but it is unlikely that the inconsistency can be explained wholly on the basis of impurity. Hopkins & Savory's [1911] value, which is in agreement with those of O[illegible]son & Nordh [1929] and of Magnus-Levy [1936], was obtained on 'practically ash-free' material dried at 110° and hence it is not to be brought into conformity with the higher value of Calvery & Freyberg [1935], as these authors suppose, by 'failure to correct for the ash and moisture content of the protein'. The S value is somewhat higher than has been obtained before but checks well with the determination of the S distribution. The values for the amino-acids also suggest that we are dealing with a protein chemically distinct from that of Calvery & Freyberg [1935]. The results of chemical analysis are therefore in agreement with evidence from other sources that Bence-Jones protein is of variable composition and that it represents a group of distinct but possibly similar proteins. There is little doubt that some of the samples which have been examined were chemically identical.

The origin of the protein remains obscure. The studies of Folin & Denis [1914], Medes *et al.* [1935] and others have shown that it cannot be considered of exogenous origin. The very considerable amount excreted in some cases precludes the possibility of its being a metabolite of the tumour. It seems more reasonable to regard it as the result either of the partial breakdown of a normal protein of higher molecular weight or of some hindrance to the complete synthesis of such a protein. In this way certain allied pathological conditions with which Bence-Jones proteinuria is associated might give rise to different products according to the response to different 'toxins'. On the basis of a chemical similarity first noted by Lüscher [1922], Calvery & Freyberg suggested that Bence-Jones protein may be derived from normal serum globulin by the latter being split into three particles of equal size; this would account for the molecular weight relation and for the excretion of Bence-Jones protein as a 'foreign' protein. This idea appears to entail the assumption that the protein is a single chemical entity of fixed composition. In view of the evidence to the contrary, the alternative suggestion demands consideration, viz. that the protein is the result of a deranged synthetic mechanism which would otherwise produce normal plasma or other proteins. This would account more easily for the production of a protein which may vary not only in chemical composition and behaviour but also in mol. wt. It is interesting to note in this connexion that according to Bonsdorff *et al.* [1938] the mol. wt. of Bence-Jones protein determined on only a few occasions as 35,000 has once given a value of 70,000 while the myeloma protein discussed by them had a mol. wt. of about 200,000, which is higher than for any of the recognized plasma proteins.

Brief mention should be made of Meyler's [1936] recent experiments on the nature of the protein from bone marrow and leucocytes which suggest that small undetected amounts of Bence-Jones (or similar) protein normally derive from these sources, but that in myelomatosis and certain other conditions the output is so magnified above the rate of destruction in the body that the protein appears in the blood and is excreted as a pathological constituent. Confirmation and further work along these lines is desirable.

The author is much indebted to Prof. H. J. Channon for laboratory facilities and materials, and also to Dr R. A. Morton and Mr T. W. Goodwin for their ready assistance in carrying out spectroscopic analyses. The work was carried out during the tenure of a whole-time grant from the Medical Research Council, to whom the author wishes to express his gratitude.

REFERENCES

Abderhalden & Rostoski (1905). *Hoppe-Seyl. Z.* **46**, 125.
Armstrong & Walker (1932). *Biochem. J.* **26**, 143.
Ayre (1938). *Biochem. J.* **32**, 1152.
Baernstein (1936, 1). *J. biol. Chem.* **115**, 25.
—— (1936, 2). *J. biol. Chem.* **115**, 33.
Bailey (1937). *Biochem. J.* **31**, 1396.
Bastian (1937). *Bull. Soc. Chim. biol., Paris*, **19**, 1298.
Bayne-Jones & Wilson (1922, 1). *Johns Hopk. Hosp. Bull.* **33**, 37.
—— —— (1922, 2). *Johns Hopk. Hosp. Bull.* **33**, 119.
Bence-Jones (1848). *Philos. Trans.* **138**, 55.
Berenblum & Chain (1938). *Biochem. J.* **32**, 295.
Bergmann (1935). *J. biol. Chem.* **110**, 471.
—— & Niemann (1936). *J. biol. Chem.* **115**, 77.
—— —— (1937). *J. biol. Chem.* **118**, 301.
—— & Stein (1939). *J. biol. Chem.* **128**, 217.
Block (1934). *J. biol. Chem.* **106**, 457.
—— (1937). *Proc. Soc. exp. Biol., N.Y.*, **37**, 580.
—— (1938). Determination of the amino-acids. Minneapolis, U.S.A.: Burgess Publishing Co.
—— (1940). *J. biol. Chem.* **133**, 67.
Bonsdorff, Groth & Packalén (1938). *Folia Haemat., Lpz.*, **59**, 184.
Calvery & Freyberg (1935). *J. biol. Chem.* **109**, 739.
Conrad & Berg (1937). *J. biol. Chem.* **117**, 351.
Davenport, Fisher & Wilhelmi (1938). *Biochem. J.* **32**, 602.
Denis (1910). *J. biol. Chem.* **8**, 427.
Engeland & Bastian (1937). *Bull. Soc. Chim. biol., Paris*, **19**, 1126.
Fisher & Wilhelmi (1937). *Biochem. J.* **31**, 1136.
Földes (1936). *Biochem. Z.* **283**, 199.
Folin & Ciocalteu (1927). *J. biol. Chem.* **73**, 627.
—— & Denis (1914). *J. biol. Chem.* **18**, 277.
—— & Marenzi (1929). *J. biol. Chem.* **83**, 89.
Guest (1939). *Canad. J. Res.* **17** (B), 143.

Hewitt (1929). *Biochem. J.* **23**, 1147.

Holiday (1936). *Biochem. J.* **30**, 1795.

Hopkins & Savory (1911). *J. Physiol.* **42**, 189.

Jervell (1932). *Biochem. Z.* **250**, 308.

Jervis, Block, Bolling & Kanze (1940). *J. biol. Chem.* **134**, 105.

Jorpes & Thorén (1932). *Biochem. J.* **26**, 1504.

Kapeller-Adler (1932). *Biochem. Z.* **252**, 185.

—— (1933). *Biochem. Z.* **264**, 131.

Kassell & Brand (1938). *J. biol. Chem.* **125**, 145.

Kekwick (1940). *Biochem. J.* **34**, 1248.

Knoop (1908). *Beitr. chem. Physiol. Path.* **11**, 396.

Koessler & Hanke (1919). *J. biol. Chem.* **39**, 497.

Kossel & Kutscher (1900). *Hoppe-Seyl. Z.* **31**, 165.

Lang (1933). *Hoppe-Seyl. Z.* **219**, 148.

Lugg (1932). *Biochem. J.* **26**, 2160.

—— (1933). *Biochem. J.* **27**, 668.

—— (1937). *Biochem. J.* **31**, 1422.

—— (1938, 1). *Biochem. J.* **32**, 775.

—— (1938, 2). *Biochem. J.* **32**, 2114.

—— (1938, 3). *Biochem. J.* **32**, 2123.

Luscher (1922). *Biochem. J.* **16**, 556.

Macara & Plimmer (1940). *Biochem. J.* **34**, 1431.

McFarlane (1935). *Biochem. J.* **29**, 1202.

— & Guest (1939). *Canad. J. Res.* **17** (B), 139.

Magnus-Levy (1936). *Hoppe-Seyl. Z.* **243**, 173.

Mainzer (1932, 1). *Biochem. Z.* **246**, 134.

—— (1932, 2). *Biochem. Z.* **246**, 149.

—— (1932, 3). *Biochem. Z.* **246**, 156.

Masters (1939). *Biochem. J.* **33**, 1313.

Medes, Berglund & Neemes (1935). The kidney in health and disease (ed. Berglund & Medes). London: H. Kimpton.

Meyler (1936). *Arch. intern. Med.* **57**, 708.

Ohlsson & Nordh (1929). *Biochem. Z.* **215**, 440.

Painter & Franke (1936). *J. biol. Chem.* **114**, 235.

Racker (1940). *Biochem. J.* **34**, 89.

Robinson (1927). *Brit. J. exp. Path.* **8**, 454.

Svedberg & Sjögren (1929). *J. Amer. chem. Soc.* **51**, 3594.

Thomas, Ingalls & Luck (1939). *J. biol. Chem.* **129**, 263.

Tristram (1939). *Biochem. J.* **33**, 1271.

Tsutsui (1935). *J. Biochem.* **22**, 343.

Waldschmidt-Leitz & Akabori (1934). *Hoppe-Seyl. Z.* **224**, 187.

Weber (1930). *J. biol. Chem.* **86**, 217.

Woolley & Peterson (1937). *J. biol. Chem.* **122**, 207.

44. DIFFUSING FACTORS

7. CONCENTRATION OF THE MUCINASE FROM TESTICULAR EXTRACTS AND FROM *CROTALUS ATROX* VENOM

BY J. MADINAVEITIA[1]

From the Chemistry Department, University of Manchester

(*Received 10 March 1941*)

FRACTIONATION of testicular extracts by lead acetate at different *p*H [Morgan & McClean, 1932; Madinaveitia, 1938] and by $(NH_4)_2SO_4$ at various concentrations [Madinaveitia, 1939, 1] are convenient methods for the concentration of diffusing factors from these sources. There is not complete parallelism between the mucolytic and diffusing activities of concentrates obtained by either of these two methods [Madinaveitia *et al.* 1940]. However, by a combination of both procedures highly active preparations of mucinase have now been obtained. The relatively accurate viscosimetric assay method [Madinaveitia & Quibell, 1940] for mucolytic activity has made it possible to determine with some exactitude the optimal *p*H range for lead acetate purification. The best mucinase preparations obtained by this method were about 1000 times more active than the dry testicle powder used as a starting material.

From a dry testicle powder *M* NaCl in *M*/10 acetic acid extracts about the same amount of mucinase as *M*/10 acetic acid alone. The precipitate formed on dilution of the NaCl extract with water contains the whole of the mucinase and the diffusing factor present in the original extract. This is probably due to adsorption of the active constituents on the protein precipitate which occurs as the electrolyte concentration is decreased. The mucinase from preparations purified with $(NH_4)_2SO_4$ does not precipitate in this way and the purest preparations of mucinase and of diffusing factor are soluble in distilled water. In this precipitation we again observe the mucinase and the diffusing factor going together in a fractionation.

The substrate used throughout this paper for testing the mucinase activity [Madinaveitia & Quibell, 1940] was a preparation of vitreous humour. Following the original idea of Chain & Duthie [1940] that diffusing factors are mucinases, Mayer *et al.* [1940, 1, 2] have suggested that the substrate on which these factors act in the skin is similar to, if not identical with, the polysaccharide from the cornea. However, intracorneal injections of diffusing factor preparations into the isolated eyes of slaughtered cattle do not increase the area of spread relative to control injections.

There is evidence that the diffusing factor and the neurotoxic constituents of *C. terrificus* are different [Madinaveitia, 1939, 2]. The mucolytic action of *C. atrox* venom has now proved to be due to a more thermolabile constituent than either the protease or the lecithinase of this venom. Fuller's earth readily adsorbs the mucinase from dialysed testicular extracts, but a relatively large amount of adsorbent is required for removal of the enzyme from solutions of *C. atrox* venom. In this case it is possible to remove part of the inactive material by adsorption at suitable *p*H with a small amount of fuller's earth and to produce a further inactive precipitate by readjustment of the *p*H. $(NH_4)_2SO_4$ fractionation can also be used for purification of the mucinase from the snake venom, the active constituents precipitating at about half-saturation.

---

[1] Beit Memorial Research Fellow.

EXPERIMENTAL

Purification of testicular mucinase

$(NH_4)_2SO_4$ fractionation. Dry testicle powder [Madinaveitia, 1938] (500 g.) mixed with sand (1 kg.) was extracted in a mortar with acetic acid (5 lots of 500 ml. *M*/10). The extract was filtered through paper and the residue twice extracted with acetic acid (2 lots of 1 l. *M*/10). To the pooled filtered extracts (4 l.; *p*H 3·7) $(NH_4)_2SO_4$ was added to 27 % of saturation. The inactive precipitate was removed and the filtrate made 70 % saturated with $(NH_4)SO_4$. The precipitate now formed contained practically the whole of the mucinase. It was dissolved in water (200 ml.) and dialysed in a cellophane bag for 24 hr. against running tap water. The dialysed mucinase solution (1 l.) was concentrated *in vacuo* (37°) and the concentrated solution (100 ml.) evaporated to dryness whilst frozen. Yield: 22 g. having 85 % of the mucinase of the original material.

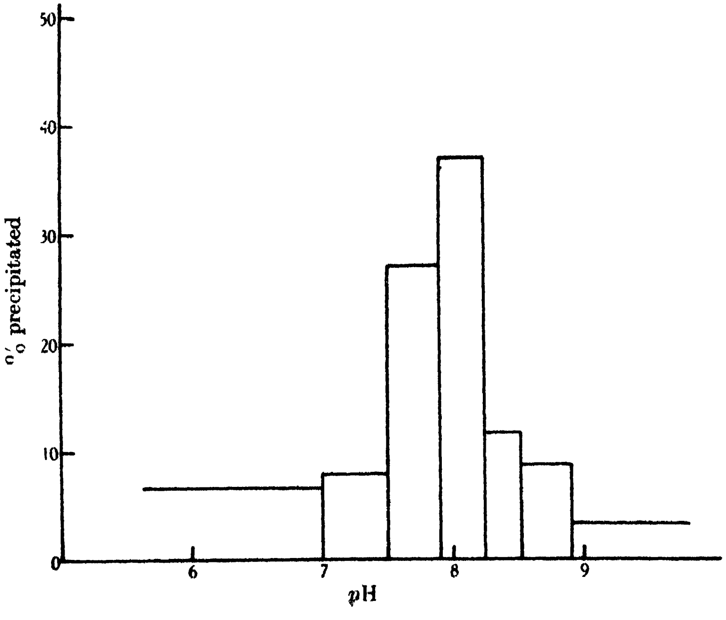


Fig. 1. Lead acetate precipitation of testicular mucinase at different *p*H.

Lead acetate fractionation. To a solution (*p*H 4·37) of $(NH_4)_2SO_4$-purified mucinase (2 g.) in water (5 ml.) a solution of neutral lead acetate (2 ml. 10 %) was added. To this opalescent mixture (*p*H 5·2) dilute ammonia (*M*/5) was added in small portions; the *p*H was determined electrometrically and the precipitate removed before addition of further amounts of ammonia. Each of the precipitates was dissolved in acetic acid (2 ml. *M*/10) and all of them made up to the same volume (50 ml.) with water. Fig. 1 represents the relative mucolytic activities of these precipitates.

A larger amount (10 g.) $(NH_4)_2SO_4$-purified mucinase was dissolved in water (250 ml.) and to the solution neutral lead acetate (100 ml. of 10 %) was added.

The pH was then adjusted to 7·37 with ammonia ($2N$) and the precipitate discarded. The filtrate was adjusted to pH 8·55 with further amounts of ammonia. The precipitate now formed was collected, dissolved in acetic acid (50 ml. $M/10$) and dialysed for 18 hr. in a cellophane bag against running tap water. After filtering from an inactive precipitate the dialysed solution was evaporated to dryness whilst frozen. The resulting preparation (0·7 g.) had 80 % of the mucinase of the already purified starting material. Lead acetate in the concentrations used in the test has no effect on the action of the mucinase on the substrate.

NaCl fractionation. Dry testicle powder (1 g.) mixed with sand (2 g.) was extracted in a mortar with a mixture of NaCl (18 ml. M) and acetic acid (2 ml. M). After 15 min. the insoluble constituents were centrifuged off. The slightly turbid supernatant (1 ml.) was diluted with water (19 ml.) and kept for 1 hr. Precipitate and supernatant were now separated and the former dissolved in NaCl (20 ml. M). Dilutions (1/20) of each of the fractions were tested for mucolytic and diffusing activities [Madinaveitia, 1938, 1939, 1].

	Mucolytic activity. % of original solution	Diffusing activity. Area of spread in cm.^{2}	Increase of the area of spread
Original extract	100	8·31	4·29
Supernatant	5	4·81	0·79
Precipitate	75	7·58	3·56
Control	-	4·02	—

To a solution of $(NH_4)_2SO_4$-purified mucinase (300 mg.) in water (12 ml.) solid NaCl (2 g.) was added. On addition of acetic acid (0·5 ml. M) a precipitate was formed (precipitate i) which, after 10 min., was collected by centrifuging. In the supernatant a further amount of NaCl (1 g.) was dissolved and the precipitate now formed (precipitate ii) collected as before. Each of the precipitates was dissolved in water (10 ml.), their N contents established and dilutions in water of each precipitate (1/10) and the final supernatant (1/50) were tested for mucolytic activity, no precipitate appearing on dilution.

	% of original solution	
	N	Mucinase
Precipitate i	28·5	5
Precipitate ii	38·5	8
Supernatant	24·5	86

Heat-inactivation of Crotalus atrox *venom*

Crude dried *C. atrox* venom (0·5 g.) dissolved in water (25 ml.) was heated for 10 min. in thermostats at different temperatures. Each sample was then made isotonic by addition of the appropriate volume of 8·5 % NaCl. The lecithinase activity was estimated by the method of Slotta & Szyszka [1938]. Proteolytic activity was then estimated by allowing the venom solution (1 ml.) to react with gelatin (10 ml. 5 %) at 37° and determining the degree of hydrolysis by formaldehyde titration.

Temperature heated °C.	Lecithinase activity. Dilution of enzyme					Proteolytic activity increase ml. $M/50$ KOH		Mucinase half life-time min.
	1/100	1/200	1/400	1/800	1/1600			
	Haemolysis					4 hr.	18 hr.	
Unheated	+	+	+	+	−	0·14	0·25	7
45°	+	+	+	+	−	0·15	0·25	14·5
55°	+	+	+	+	−	0·12	0·25	∞
65°	+	±	±	−	−	0·14	0·25	—

Purification of the mucinase of Crotalus atrox *venom*

Solutions of the venom (12 mg.) in water (12 ml.) are turbid. The insoluble material is brought into solution by addition of acetic acid (*M*/10), Na_2HPO_4 (*M*/15) and to a less extent by NaCl (*M*/3). The water-insoluble constituents are inactive and readily flocculated at *p*H 5·1 (*M*/15 acetate buffer).

$(NH_4)_2SO_4$ fractionation. A solution of the dry crude venom (1·65 %) was centrifuged and to the supernatant (4 ml.) acetic acid (0·2 ml. *M*) were added. To this solution (2 ml.) increasing amounts of saturated $(NH_4)_2SO_4$ solution were added, the precipitate being removed after each addition. The precipitates were dissolved in *M* NaCl (10 ml.) and tested for mucolytic activity:

% saturation in $(NH_4)_2SO_4$	0–33	33–50	50–67
Mucolytic activity % of original solution	21	62	25

Dry *C. atrox* venom (1 g.) dissolved in water (50 ml.) was acidified with acetic acid (2·5 ml. *M*); saturated $(NH_4)_2SO_4$ (25 ml.) was added and the precipitate discarded. To the clear solution more $(NH_4)_2SO_4$ (75 ml. saturated solution) was added and the active precipitate collected. This precipitate was extracted with water (20 ml.), the extract acidified with acetic acid (0·2 ml. *M*) and again precipitated with $(NH_4)_2SO_4$ (5 ml. saturated solution). The inactive precipitate was discarded and the clear solution treated with more $(NH_4)_2SO_4$ (30 ml. saturated solution). The active precipitate now formed was collected, dissolved in water (25 ml.) dialysed in a cellophane bag against running tap water overnight and dried whilst frozen. Yield: 0·07 g. of a preparation having 40 % of the mucinase content of the starting material (1 g. crude venom).

Adsorption of C. atrox *mucinase.* A solution (0·5 %) of crude venom was centrifuged. To part of the supernatant (20 ml.) acetic acid (1 ml. *M*) was added (*p*H 3·5) and another portion (20 ml.) was diluted with water (1 ml.) (*p*H 6·05). Samples (0·5 ml.) of each of these two solutions were shaken with different adsorbents (0·2 g.). After 30 min. the adsorbates were centrifuged and the N and mucinase contents of the supernatant estimated:

		% of original solution	
*p*H	Adsorbent	N	Mucinase
3·5	Kaolin	28	39
	Kieselguhr	34	18
	Fuller's earth	48	12
6·0	Kaolin	41	0
	Kieselguhr	34	10
	Fuller's earth	48	10

Adsorption with fuller's earth. A centrifuged solution (50 ml. 0·5 %) of crude *Crotalus* venom was acidified with acetic acid (1·25 ml. *M*). Samples of the solution (5 ml.) were shaken with various amounts of fuller's earth and kept for 30 min. with occasional stirring. After removal of the adsorbates the N and mucinase contents of the supernatants were estimated:

g. Fuller's earth	0·8	0·4	0·2
% N adsorbed	96	77	48
% Mucinase adsorbed	100	66	25

To samples (5 ml.) of a solution of crude venom (5 mg./ml.) acetate buffers (1 ml. *M*) of different *p*H were added and each sample adsorbed with fuller's earth (0·25 g.). The adsorbate was removed and dilutions (1/3) of the supernatant in *M* citrate buffer *p*H 4·7 tested for mucolytic activity. The N contents of the supernatants were also determined. The results are recorded in Fig. 2.

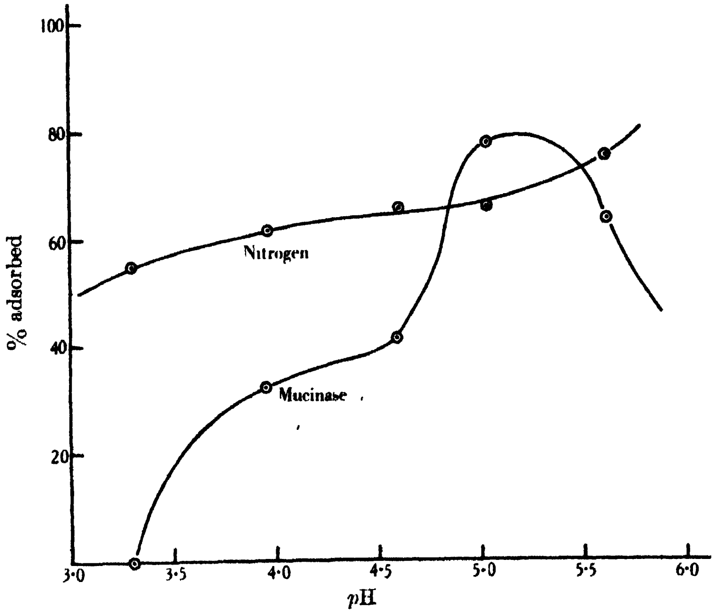


Fig. 2. Effect of *p*H on the adsorption of snake venom mucinase on fuller's earth.

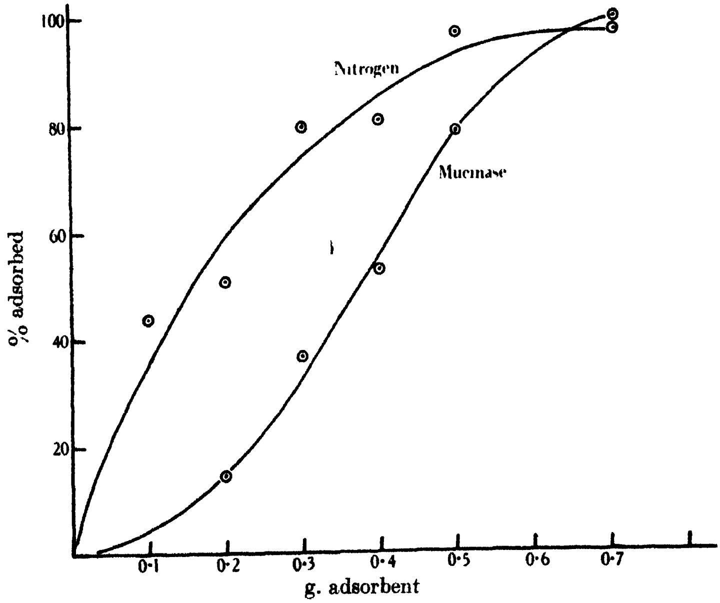


Fig. 3. Effect of the amount of adsorbent on the adsorption of snake venom mucinase on fuller's earth.

A solution (5 mg./ml.) of crude venom was mixed with acetate buffer (*M*). Samples of the buffered solution (*p*H 3·7) were adsorbed with various amounts of fuller's earth and after 30 min. the adsorbates were removed. Dilutions (1/3) of the supernatant in *M* citrate buffer *p*H 4·7 were tested for mucolytic activity, and the N contents of the supernatants determined. The results are recorded in Fig. 3.

A solution of crude venom (60 ml., 5 mg./ml.) was mixed with acetate buffer (5 ml. *M*). The mixture (*p*H 3·5) was shaken with fuller's earth (2·5 g.) and filtered after 15 min. Samples (10 ml.) of the filtrate were partially neutralized with NaOH (1 ml.) of increasing concentration. The precipitate now formed was centrifuged after 1 hr. and the amounts of mucinase and N precipitated were determined.

	*p*H ...	4·50	5·25	6·00
% precipitated:	Mucinase	0	0	20
	N	50	50	70

SUMMARY

$(NH_4)SO_4$-fractionation of aqueous testicular extracts, followed by lead acetate precipitation under controlled *p*H, gives a convenient purification of mucinase.

This enzyme can also be purified by dilution of NaCl extracts of testicle with water. Both mucolytic and diffusing activities are in the precipitate. The lecithinase and protease of *Crotalus atrox* venom are more thermostable than the mucinase. Concentration of the mucinase from this venom has been carried out by $(NH_4)_2SO_4$-fractionation and by adsorption methods.

The author's thanks are due to Prof. A. R. Todd for his continued interest and advice. He is also grateful to Imperial Chemical Industries, Ltd., for grants.

REFERENCES

Chain & Duthie (1940). *Brit. J. exp. Path.* **21**, 324.
Madinaveitia (1938). *Biochem. J.* **32**, 1806.
— — (1939, 1). *Biochem. J.* **33**, 347.
— — (1939, 2). *Biochem. J.* **33**, 1470.
—— & Quibell (1940). *Biochem. J.* **34**, 625.
— Todd, Bacharach & Chance (1940). *Nature, Lond.*, **146**, 197.
Mayer & Chaffee (1940, 1). *Proc. Soc. exp. Biol., N.Y.*, **43**, 487.
—— Hobby, Chaffee & Dawson (1940, 2). *Proc. Soc. exp. Biol., N.Y.*, **44**, 294.
Morgan & McClean (1932). *J. Soc. chem. Ind., Lond.*, **51**, 912.
Slotta & Szyszka (1938). *Ber. dtsch. chem. Ges.* **71**, 258.

45. STUDIES ON DIFFUSING FACTORS

8. THE REDUCTION OF THE VISCOSITY OF VITREOUS HUMOUR PREPARATIONS BY ASCORBIC ACID AND SOME DIAZO COMPOUNDS

By J. MADINAVEITIA[1] AND T. H. H. QUIBELL

From the Chemistry Department, University of Manchester

(*Received 10 March 1941*)

THE work of McClean & Hale [1940] and of Favilli [1940] indicates that the viscosity of vitreous humour preparation is decreased not only by the diffusing factors of biological origin, but also by some compounds known to increase the area of spread of indicators when tested for diffusing activity. The two types of compounds examined have been ascorbic acid and diazo compounds.

It has been established [Madinaveitia & Quibell, 1940] that, under certain conditions, the concentration of testicular extract or snake venom required to bring about a given change of the viscosity of a preparation of vitreous humour is inversely proportional to the time required for this change to take place. The effect of the concentration of ascorbic acid on the time required to decrease by a given amount the viscosity of a vitreous humour preparation does not follow such a simple law as testicular extracts of snake venom (Fig. 1). It is therefore unlikely that the mechanism of the reaction in which the viscosity is lost is the same in the case of ascorbic acid as in those of preparations from testicle or snake venom.

The spreading properties of diazobenzene-*p*-sulphonic acid and of some proteins coupled with it have been known for some time [Claude, 1933; 1935]. As reported by Favilli [1940] diazobenzene-*p*-sulphonic acid also causes a reduction in the viscosity of mucin preparations. The same effect has now been observed with diazotized aniline. Aniline, sulphanilic acid and sodium nitrite are without effect. When diazobenzenesulphonic acid is coupled with either dimethylaniline or with phenol the resulting dyestuff has no effect on the viscosity of vitreous humour preparations.

It is difficult to picture the type of reaction which occurs between the diazo compound and the viscous material which is of a polysaccharide nature, built up of acetyl glucosamine and uronic acid residues [Palmer *et al.* 1937]. Phenylhydrazine reacting with α-aminocarbonyl compounds will form osazones [Fischer, 1893], in some cases even when the nitrogen is substituted [Jacob & Madinaveitia, 1937]. Similarly it has been found in these Laboratories by Miss M. East that phenylhydrazine reacts with *N*-acetyl glucosamine yielding glucosazone. A slow reduction of the viscosity of vitreous humour preparations is observed when phenylhydrazine acts on them. In the reacting mixture the presence of diazo compounds can be detected. Since the reaction is greatly accelerated by H_2O_2 (Fig. 2) it seems likely that small amounts of diazobenzene formed by the oxidation of phenylhydrazine are responsible for the decrease in the viscosity. Neither semicarbazide nor hydroxylamine have any effect on the viscosity of vitreous humour preparations so that the action of phenylhydrazine cannot be due merely to its property of reacting with carbonyl groups.

---

[1] Beit Memorial Research Fellow.

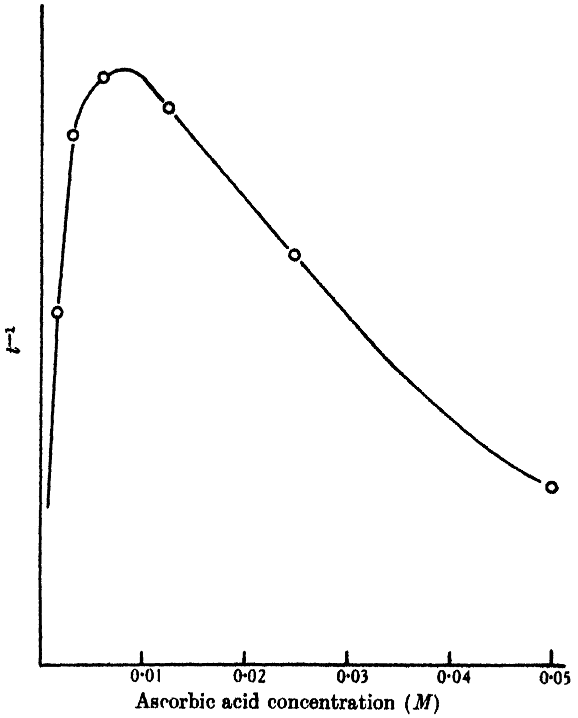
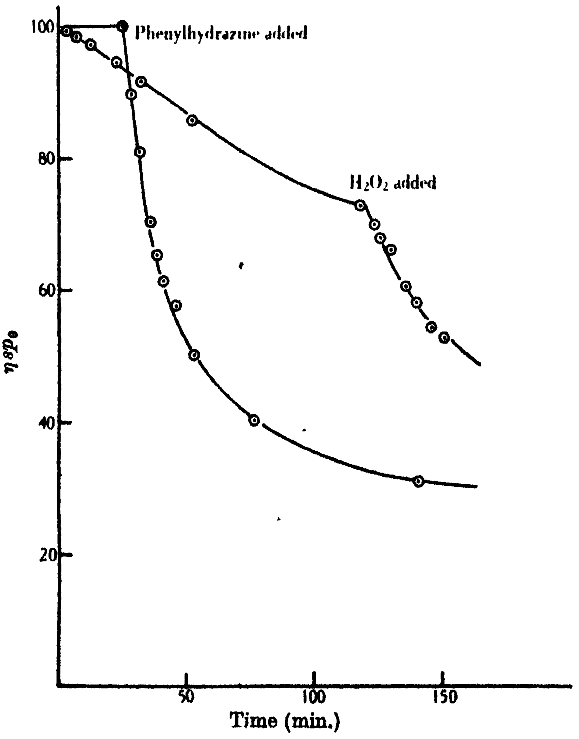


Fig. 1. Effect of the concentration of ascorbic acid on the rate of decrease of the viscosity of vitreous humour preparations.



Action of phenylhydrazine and H_2O_2 on the viscosity of vitreous humour.

EXPERIMENTAL

Action of ascorbic acid. Ascorbic acid (1·7 g.) dissolved in *M* citrate buffer *p*H 4·7 (15 ml.) was partially neutralized with *M* NaOH (5 ml.). Dilutions of the solution in *M* citrate buffer (1 ml.) were allowed to react with a solution of a vitreous humour acetone powder (4 ml.) in *M*/5 citrate buffer having *M* NaCl in a viscosimeter in a thermostat (40°). The technique used for following the fall of viscosity was that previously described [Madinaveitia & Quibell, 1940]. The results obtained are plotted in Fig. 1.

*Action of diazobenzene-*p*-sulphonic acid.* An ice-cold suspension of the diazo compound in water (2 ml.) was mixed with chilled *M* NaOH (2 ml.) and *M* citrate buffer *p*H 4·7 (2 ml.) was added to the mixture. This solution (1 ml.) was further diluted with citrate buffer which was *M*/2 in NaCl. A solution of a vitreous humour preparation (see above) (5 ml.) was allowed to react with the diluted solution of the diazo compound (1 ml.) at 25°.

Action of aniline and diazobenzene. A vitreous humour solution as used in the previous experiment (5 ml.) was mixed with *M*/5 aniline hydrochloride (0·5 ml.). The viscosity remained unchanged for 20 min., when *M*/5 $NaNO_2$ (1 ml.) was added. The viscosity now decreased at a speed comparable with that observed with diazobenzene-*p*-sulphonic acid.

Action of phenylhydrazine. Freshly distilled phenylhydrazine (1 ml.) was dissolved in *M* citrate buffer *p*H 4·7 (29 ml.). A sample of this solution (1 ml.) was allowed to react with a solution of vitreous humour acetone powder (5 ml.) in buffered *M* NaCl at 25°. The viscosity slowly decreased. After 1 hr. a small amount (0·05 ml.) was removed and acidified with HCl. On addition of a solution of α-naphthylamine in HCl a pink colour developed. A control test with phenylhydrazine solution alone was negative.

A buffered solution of vitreous humour (5 ml.) was mixed with the above phenylhydrazine solution (1 ml.). After the mixture had stood for 2 hr. at 25° H_2O_2 (0·1 ml. 30 %) was added. Now the viscosity rapidly decreased (Fig. 2). The same vitreous humour solution (5 ml.) was mixed with 30 % H_2O_2 (0·5 ml.). The viscosity of the mixture did not decrease with time, but when phenylhydrazine solution (1 ml.) was added the viscosity at once started to decrease (Fig. 2).

SUMMARY

The observations of McClean & Hale [1940] and of Favilli [1940] that ascorbic acid and diazo compounds produce a gradual diminution of the viscosity of mucin preparations have been confirmed. The effect of the concentration of ascorbic acid on the time required to bring about a given decrease of the viscosity of vitreous humour preparations does not follow the same law as testicular extracts or snake venom. Phenylhydrazine has the effect of lowering the viscosity of mucins in a similar way. The action is much more marked in the presence of H_2O_2. Other carbonyl reagents are inactive.

REFERENCES

Claude (1933). *Science*, **78**, 151.
—— (1935). *J. exp. Med.* **62**, 229.
Favilli (1940). *Nature, Lond.*, **145**, 866.
Fischer (1893). *Ber. dtsch. chem. Ges.* **26**, 95.
Jacob & Madinaveitia (1937). *J. chem. Soc.* p. 1929.
Madinaveitia & Quibell (1940). *Biochem. J.* **34**, 65.
McClean & Hale (1940). *Nature, Lond.*, **145**, 866.
Palmer, Smith & Meyer (1937). *J. biol. Chem.* **119**, 491.

46. DIFFUSING FACTORS

9. THE EFFECT OF SALTS ON THE ACTION OF TESTICULAR EXTRACTS ON THE VISCOSITY OF VITREOUS HUMOUR PREPARATIONS

By J. MADINAVEITIA[1] and T. H. H. QUIBELL

From the Chemistry Department, University of Manchester

(*Received 10 March 1941*)

The reduction of the viscosity of certain mucins by testicular extracts and by mucinases from other sources of diffusing factors has been associated by Chain & Duthie [1939] with the characteristic spreading properties of these factors. Comparison of the relative diffusing and mucolytic activities of diffusing factor preparations [Madinaveitia *et al.* 1940] requires a relatively accurate quantitative estimation of the two effects. Methods are now available which permit such a comparison to be carried out; the diffusing activity can be determined with enough accuracy by the technique of Bacharach *et al.* [1940] and the available viscosimetric method [Madinaveitia & Quibell, 1940] accurately measures mucolytic activity. The results obtained by these methods do not quite agree with the view that the effect of diffusing factors is solely due to the mucolytic activity of the assayed preparations.

The viscosimetric method for the estimation of mucinases is based on the fact that the rate of decrease of the viscosity of vitreous humour preparation is proportional to the concentration of enzyme present in the reacting mixture, provided that the reaction is carried out under certain conditions. The mucolytic activities of a number of diffusing factor preparations have now been compared by this method. The results obtained are in good agreement when comparison is made at different concentrations of enzyme (c) after the original viscosity of the substrate (ηsp_0) has been reduced to a given value ($\%\eta sp_0$). The product of the concentration of enzyme and the time (t) required to reach the chosen viscosity level ($c \times t$) is practically constant for each preparation whatever is the decrease in the viscosity, provided that it is the same for all the samples compared (Table 1).

Table 1

Preparation	c mg./6 ml.	$\%\eta sp_0$			
		64·8	57·7	48·7	40·8
		$c \times t$			
Glaxo standard	0·49	122	196	343	538
	0·37	140	229	370	573
	0·245	134	238	368	587
Madinaveitia's [1938] standard	2·55	1120	1730	2670	4450
	1·915	950	1650	2680	4600
Crude *Crotalus atrox* venom	4·84	1350	2410	3950	5550
	2·41	1570	2340	3740	6620
Purified *Crotalus atrox* venom	1·08	194	335	540	840
	0·54	232	365	567	890
	0·36	254	397	630	950

---

[1] Beit Memorial Research Fellow.

Using one preparation as a standard of reference, the relative mucolytic activity of each of the others is the same irrespective of the viscosity level at which comparison is carried out (Table 2).

Table 2

	$\%\eta sp_0$			
	71·5	63·5	45·0	35·0
Preparation	Ratio to Glaxo Standard			
From testicle:				
Glaxo standard	100	100	100	100
Madinaveitia standard	23·5	24·7	23·2	24·2
M/10 acetic acid extract	84	85	79	67?
$(NH_4)_2SO_4$-purified	150	152	147	147
From *Crotalus atrox*:				
Crude venom	19·9	17	15·7	15·9
$(NH_4)_2SO_4$-purified venom	51·5	57·5	67	69

All comparisons were carried out under the conditions originally suggested [Madinaveitia & Quibell, 1940], i.e. in 0·66 *M* NaCl and in the presence of 0·18 *M* citrate buffer *p*H 4·7. However, on testing by this method a mucinase preparation from *Clostridium welchii* prepared by Dr D. McClean of the Lister Institute we found that contrary to his indications it was practically inactive. This apparent discrepancy was due to the fact that Dr McClean's estimation of the mucolytic activity of this preparation had been carried out in the absence of NaCl. In view of this an investigation has now been carried out on the effect of salts on the reaction in which the viscosity of vitreous humour preparations is destroyed by the action of testicular extracts. A summary of the results has already been published [Madinaveitia & McClean, 1940]. The substrate used was prepared from an acetone powder of bovine vitreous humour (1 g.) by extraction with *M*/50 NaOH (50 ml.) and precipitation of the centrifuged and acidified (10 ml. *M* acetic acid) extract with 6 vol. acetone. After washing the precipitate with ether and drying *in vacuo* a white powder was obtained which was almost completely soluble in water but which still contained some salts. The source of enzyme was a preparation obtained by $(NH_4)_2SO_4$-fractionation from an acetic acid (2 l. *M*/10) extract of dry testicle powder (100 g.). The material precipitated between 30 and 70 % of saturation was redissolved, dialysed, subsequently electrodialysed until the conductivity was practically constant and finally dried whilst frozen. It was salt-free. The techniques used for estimating the viscosity and for determining the half-life time of the viscous substrate were those already described [Madinaveitia & Quibell, 1940].

As previously reported addition of salts has the effect of decreasing the specific viscosity (ηsp_0) of the substrate (Tables 3 and 5). However, the relationship between specific viscosity and the concentration of the substrate is the same at any given NaCl concentration as indicated by the fact that the ratio of the viscosity of the substrate (ηsp_0) to that of the substrate diluted with 1 vol. of the corresponding NaCl solution ($\eta sp_{\frac{1}{2}}$) is constant (Table 3). The only ratio which differs significantly from the others is that in which there is no NaCl present but this can be easily accounted for by the fact that the vitreous humour preparation used was not absolutely free of electrolytes. Reduction of the concentration of electrolyte on dilution with distilled water would render the more diluted substrate somewhat more viscous than expected. Electrodialysis throws the viscous materials out of solution.

When a series of samples of electrodialysed enzyme (0·5 mg. in 1 ml. water) are allowed to react with a substrate solution (5 ml. 2 %) in NaCl of increasing concentration the half-life time (t) of the viscous material decreases as the concentration of NaCl in the reacting mixture becomes larger and after reaching a minimum it increases again (Table 3). Therefore there is an optimum salt concentration for the activity (t^{-1}) of the enzyme.

Table 3

NaCl M	ηsp_0	ηsp_t	$\frac{\eta sp_0}{\eta sp_t}$	t min.	t^{-1}
0·00000	1·44	0·752	1·92	60	0·012
0·00083	1·28	0·596	2·14	30	0·033
0·00167	1·12	0·513	2·18	15·5	0·064
0·00334	0·94	0·443	2·13	12·5	0·080
0·00500	0·850	—	—	12·5	0·080
0·00667	0·775	—	—	9·0	0·112
0·00833	0·725	0·339	2·14	6·0	0·167
0·01677	0·593	0·279	2·13	4·2	0·238
0·0334	0·491	0·234	2·10	3·3	0·303
0·0500	0·441	—	—	2·3	0·425
0·0667	0·417	—	—	0·7	1·43
0·0833	0·400	—	—	0·7	1·43
0·167	0·339	—	—	0·7	1·43
0·334	0·308	—	—	3·0	0·333
0·500	0·289	—	—	3·2	0·312
0·667	0·283	—	—	4·7	0·213
0·833	0·270	—	—	14·5	0·069
1·670	0·259	—	—	30	0·033

The same effect was observed when a similar experiment was carried out in the presence of 0·2M citrate buffer pH 4·7 and with concentrations of substrate and of enzyme different from those in the previous experiment. The same picture is obtained at whatever fraction of the original viscosity the comparison is carried out (Table 4). In addition to NaCl certain other salts in dilute solution also enhance the action of the enzyme and all the salts examined inhibit at relatively high concentrations. In Table 5 the effect of increasing amounts of NaCl is compared with that of other salts. The reaction was carried out in the presence of 0·2M Na citrate buffer pH 4·7.

Table 4

NaCl	$\% \eta sp_0$					
M	65	60	55	50	45	40
	Sec. required to reach $\% \eta sp_0$					
0·00	300	380	900	1580	2400	3500
0·01	190	240	310	440	700	1050
0·02	160	240	350	530	880	1450
0·05	100	190	320	500	750	1050
0·10	130	210	370	550	820	1250
0·16	110	240	330	480	700	1050
0·21	130	240	370	550	820	1250
0·53	150	290	500	850	1320	2100
1·56	170	1050	1900	—	—	—
2·08	1300	2900	5000	—	—	—

The method suggested [Madinaveitia & Quibell, 1940] for the comparison of the activities of mucinase preparations depends on the half-life time of the substrate being inversely proportional to the concentration of enzyme. This relationship does not hold good when working in an unbuffered solution at low

Table 5

	t	ηsp_0	t	ηsp_0	t	ηsp_0
Without salt	15	0·515	15	0·515	15	0·515
Molarity of salt	0·0083		0·083		0·83	
NaCl	7·5	0·430	7	0·392	29·5	0·330
KCl	7·5	0·435	7	0·402	29·5	0·326
NH_4Cl	5·5	0·472	5·5	0·458	45	0·310
NaI	11·5	0·404	13·5	0·382	100	0·325
NaBr	9·5	0·418	9·5	0·398	95	0·320
Na_2SO_4	15	0·422	40	0·407	200	0·450

NaCl concentrations. Under these conditions, when the concentration of enzyme is increased, the half-life time of the substrate decreases much more rapidly than expected (Fig. 1). This effect is most marked in the absence of NaCl and it becomes less noticeable as the concentration of salt increases. After a certain level of salt concentration has been reached the half-life time of the substrate becomes inversely proportional to the concentration of enzyme and thereafter this relationship is maintained although the reaction velocity decreases.

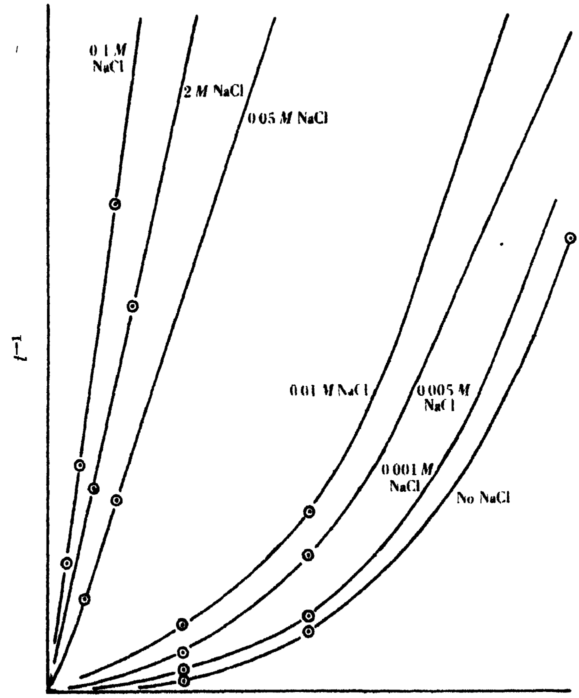


Fig. 1. Effect of the concentration of NaCl on the dose-response curve of testicular mucinase.

Testicular mucinase is not the only enzyme whose action is enhanced by the presence of salts. The effect of neutral salts on the action of different amylases is well known and has been studied in some detail [cf. Myrbäck, 1926; Sherman *et al.* 1928]. It has been found that some ions increase the activity of amylases, changing the *p*H optimum. An alteration of the kinetics of the reaction between amylase and starch due to the presence of NaCl, such as observed with testicular mucinase, has never been recorded as far as we are aware.

SUMMARY

The time required to reduce the viscosity of vitreous humour preparations to half its original value is inversely proportional to the concentration of testicular mucinase present in the reacting mixture, provided that the reaction is carried out in the presence of NaCl. At low concentrations of salt this time decreases very rapidly as the concentration of enzyme increases.

The authors' thanks are due to Prof. A. R. Todd for his continued interest and advice. They are also grateful to Dr D. McClean of the Lister Institute for supplies of bacterial filtrates and much useful discussion. Grants from Imperial Chemical Industries are gratefully acknowledged.

REFERENCES

Bacharach, Chance & Middleton (1940). *Biochem. J.* **34**, 1464.
Chain & Duthie (1939). *Nature, Lond.* **144**, 977.
Madinaveitia (1938). *Biochem. J.* **32**, 1470.
—— & McClean (1940). *J. Soc. Chem. Ind., Lond.* **59**, 418.
—— & Quibell (1940). *Biochem. J.* **34**, 625.
—— Todd, Bacharach & Chance (1940). *Nature, Lond.* **146**, 197.
Myrbäck (1926). *Hoppe-Seyl. Z.* **159**, 1.
Sherman, Caldwell & Adams (1928). *J. Amer. chem. Soc.* **50** 2535.

47. ESTERS OF PHOSPHORIC ACID

4. PHOSPHORYL HYDROXYAMINO-ACIDS

By R. H. A. PLIMMER

From the Chemistry Department, St Thomas's Hospital Medical School, London, S.E.

(*Received 12 March 1941*)

PHOSPHOTYROSINE was made by Levene & Schormüller [1933] by the action of $POCl_3$ in CCl_4 on formyltyrosine in presence of MgO. From the Mg salt of formylphosphotyrosine the formyl group was removed by hydrolysis with HCl and phosphotyrosine was obtained through the Ba and Pb salts.

Levene & Schormüller [1934, 1] tried three methods of preparing phospho-*dl*-serine of which only direct phosphorylation with H_3PO_4 and P_2O_5 was successful. The Ba salt was isolated. Later Levene & Schormüller [1934, 2] prepared larger quantities and separated the stereoisomers by fractional crystallization of the brucine salts. They also isolated the Ba salt of phospho-*l*-hydroxyproline after direct phosphorylation. Phosphohydroxyaspartic acid and phosphohydroxyglutamic acid could not be made. Neither phosphoserine nor phosphohydroxyproline were prepared and very little information was given of the properties of the compounds. Further information was desirable, especially concerning the hydrolysis of these esters by acid, alkali and phosphatase to compare with caseinogen, caseo-phosphopeptone and other esters of phosphoric acid.

EXPERIMENTAL

1. *Phosphotyrosine*

The successful phosphorylation of amino-ethanol and choline by Plimmer & Burch [1937] by heating the substances with H_3PO_4 and P_2O_5 led to a trial with tyrosine. On direct heating, the mixture darkened and effervesced, but it was possible to isolate a small yield of the Ca salt of phosphotyrosine. Subsequent experiments were made by heating the mixture (2–5 g. tyrosine, 14–35 g. H_3PO_4 and 2–5 g. P_2O_5) in a small flask with $CaCl_2$ tube on a water bath for 1, 6, 48, 64 hr. to ascertain the condition for the best yield. The resulting syrup was dissolved in water and made to 500 ml. and an aliquot was taken for N determination to give information of the amount of substance in later solutions. The solution was made alkaline to phenolphthalein with a fine suspension of $Ca(OH)_2$, or with $Ba(OH)_2$ solution, and filtered from $Ca_3(PO_4)_2$ and excess $Ca(OH)_2$, or $Ba_3(PO_4)_2$, which was washed once or more with water until all the N was in solution. In one experiment the Ca salt was isolated by concentrating *in vacuo* and adding an equal volume of absolute alcohol. The air-dried salt was heated at 100° *in vacuo* over P_2O_5 for analysis. (Found: N, 4·48; P, 10·02; Ca, 13·55; H_2O, 14·53 %. $C_9H_{10}O_6NPCa$ requires N, 4·68; P, 10·36; Ca, 13·40; $+3H_2O$ requires H_2O, 15·30 %.) The low water content is probably due to diffculty in removing water from phosphoric esters. Some $CaCO_3$ was present.

In another experiment the Ba salt was isolated by adding an equal volume of absolute alcohol to the concentrated filtrate. It came down as a very fine precipitate; if kept in air it became syrupy and set to a glass which was easily powdered. It was redissolved in water, reprecipitated and dried in a desiccator. For analysis, it was heated at 100° *in vacuo* over P_2O_5. (Found: N, 3·58; P, 7·48; Ba, 35·22; H_2O, 13·22 %. $C_9H_{10}O_6NPBa$ requires N, 3·53; P, 7·79; Ba, 34·65; $+4H_2O$ requires H_2O, 15·38 %.)

Again, the difficulty in removing H_2O from phosphoric esters will account for the low H_2O value. $BaCO_3$ was also present.

In other experiments lead acetate solution was added to the filtrate until there was no further precipitation. The Pb salt, after washing, was suspended in water and decomposed with H_2S and the filtrate from PbS concentrated and treated with an equal volume of absolute alcohol. The resulting crystals were filtered off and washed with 50 % alcohol. Further crops of phosphotyrosine were obtained on concentrating the solution and adding alcohol. The crops crystallizing out first consisted of nearly pure phosphotyrosine; later crops were admixed with tyrosine. The pure compound was obtained by redissolving, filtering and adding alcohol. The several preparations dried at 100° *in vacuo* over P_2O_5 gave on analysis: N, 5·32, 5·42, 5·41, 5·20; P, 11·92, 12·13, 11·89, 11·68 %. $C_9H_{12}O_6NP$ requires N, 5·34 %, P, 11·83 %.

Phosphotyrosine crystallizes in shining platelets; on heating it shrinks at 224° and melts at 225°. It dissolves slowly in cold water, more rapidly on warming. 0·2584 g. in 25 ml. 2*N* HCl gave in a 2 dm. tube a rotation of −0·19°, $[\alpha]_D - 9{\cdot}19°$. These data do not agree with those of Levene & Schormüller [1933] who gave M.P. 253° and $[\alpha]_D - 2{\cdot}0°$.

A 0·6 % solution of phosphotyrosine in water gave precipitates only with Pb acetate and $HgNO_3$ solutions. The other salts are thus soluble in water. The Pb salt, isolated in one preparation, dried in air and heated at 100° *in vacuo* over P_2O_5, gave on analysis: N, 2·93; P, 6·86; Pb, 43·74; H_2O, 3·88 %. $C_9H_{10}O_6NPPb$ requires N, 3·00; P, 6·65; Pb, 44·42; $+H_2O$ requires H_2O, 3·72 %.

Phosphotyrosine does not give a blue colour with Folin's phenol reagent. With Millon's reagent at room temperature it turns brown on standing; on warming there is slow development of colour, probably due to hydrolysis to tyrosine.

The yields of phosphotyrosine were 28 % on heating for 1 hr.
41 % „ 6 hr.
48 % „ 48 hr.
55 % „ 64 hr.

the extra time thus being advantageous.

2. *Phosphohydroxyproline*

l-Hydroxyproline (Roche) was phosphorylated in the same way as tyrosine with 2 g. at a time. The syrupy residue was dissolved in water, and the solution treated with $Ba(OH)_2$ solution, or $Ca(OH)_2$ suspension. The filtrate and washings from the precipitate were concentrated *in vacuo* and precipitated with alcohol. The yield of Ba salt was 48 % on phosphorylating for 24 hr. and 51 % after 40 hr. At room temperature the phosphorylation was 26 % in 13 days. The Ba salt was dried in air and heated at 100° *in vacuo* over P_2O_5 for analysis. (Found: N, 3·46; P, 8·81; Ba, 39·23; H_2O, 16·24 %. $C_5H_8O_6NPBa$ requires N, 4·05; P, 8·96; Ba, 39·50; $+4H_2O$ requires H_2O, 17·23 %.)

If precipitated from hot solution, H_2O was 3·50 to 3·88 %, and the salt dried at 100° *in vacuo* over P_2O_5 showed N 3·9, P 8·5, Ba 36·6 %, indicating $1H_2O$ and partial conversion into acid salt.

The Pb salt was prepared from the Ba or Ca salt by precipitation with Pb acetate. After filtration and washing it was suspended in water and decomposed with H_2S. The filtrate from PbS was evaporated *in vacuo* to a small volume and alcohol added in small portions. Phosphohydroxyproline came down as an oil, which could be hardened with alcohol and became crystalline, or it could be obtained crystalline directly by very slowly adding alcohol and scratching.

The analyses of the several specimens of phosphohydroxyproline presented difficulty in the interpretation of the results:

On heating the air-dried substance at 100° *in vacuo* over P_2O_5 and analysing the residue:

H_2O varied from 4·96 to 5·75 %;

N varied from 5·81 to 6·11 %; average 6·00 %;

P varied from 13·46 to 14·00 %; average 13·70 %.

The most usual figures were N, 6·10; P, 13·58 %.

$C_5H_{10}O_6NP$ requires N, 6·63; P, 14·69 %. $C_5H_{10}O_6NP$, H_2O requires N, 6·11; P, 13·54; H_2O, 7·86 %.

The air-dried substance showed N, 5·66; P, 13·28 %.

$C_5H_{10}O_6NP$, $1{\cdot}5H_2O$ requires N, 5·88; P, 13·03 %.

The loss of H_2O on heating at 100° is thus between 1·5 and $1H_2O$.

Not until the substance was heated to 130° *in vacuo* over P_2O_5 was the water of crystallization completely driven off. (Found: H_2O, 10·91; N, 6·66; P, 14·96 %. On exposing the dried substance to air it took up H_2O, 7·71 %.)

The air-dried substance would thus be $C_5H_{10}O_6NP$, $1{\cdot}5H_2O$; after heating to 100° $C_5H_{10}O_6NP$, H_2O; after heating to 130° $C_5H_{10}O_6NP$.

Phosphohydroxyproline crystallizes in fine needles, M.P. 115°. The anhydrous substance melts at 130–131° with frothing but without decomposition. 0·2738 g. dissolved in 15 ml. H_2O (1·825 %) in a 2 dm. tube gave a rotation of $-1{\cdot}05°$, $[\alpha]_D - 28{\cdot}76°$. The solution gave only an insoluble lead salt.

3. *Phosphoserine*

Poor yields of the Ba salt of phosphoserine were obtained by Levene & Schormüller [1934, 1, 2] by the interaction of serine, H_3PO_4 and P_2O_5 for 40 hr. at room temperature: thus 1·85 g. from 4 g. serine = approx. 15 %, and 106 g. from 200 g. serine = approx. 17 %.

As good yields of phosphotyrosine and phosphohydroxyproline were obtained on heating at 100°, the same procedure of heating 1 g. serine, 7 g. H_3PO_4 and 1 g. P_2O_5 was tried. There was no product either on heating for 24 or 45 hr. On keeping the mixture for long periods of 4, 24 and 46 days yields of Ba salt amounting to 0·5, 16 and 26 % resulted. It was possible therefore that any phosphoserine formed at 100° was hydrolysed.

Heating in an autoclave at 20 lb. pressure for 1–1·5 hr. gave yields of Ba salt varying from 8 to 21 %, no improvement on the yields at room temperature. An advantage is that the preparation takes less time.

The isolation of the Ba or Ca salt was effected in the usual way. The specimens of Ba and Ca salts after drying in air were heated at 100° *in vacuo* over P_2O_5 for analysis. (Found: N, 4·27; P, 9·63; Ba, 42·62; H_2O, 14·69 %. $C_3H_6O_6NPBa$ requires N, 4·37; P, 9·67: Ba, 42·87; $+3H_2O$ requires H_2O, 14·43 %.)

Other preparations contained $BaCO_3$.

The Ba salt of serine could be recovered from the preparations in which there was no phosphorylation and also from the filtrates from Ba phosphoserine. This salt is less soluble in water than expected.

The Pb salt of phosphoserine was precipitated on adding basic Pb acetate to the solution of the Ba salt. The air-dried salt was heated to 100° *in vacuo* over P_2O_5 for analysis. (Found: N, 1·86; P, 4·39; Pb, 61·91; H_2O, 6·09 %. It appears to be a basic salt: $C_3H_6O_6NPPb$. $Pb(OH)_2$ requires N, 2·22; P, 4·91; Pb, 65·61; $+3H_2O$ requires H_2O, 7·88 %.)

Phosphoserine was prepared from the Pb salt in the usual way by precipitating with 3 to 4 vol. alcohol. Only three specimens, amounting to 0·2, 0·14 and 0·3 g., were made. The air-dried substances heated at 100° *in vacuo* over P_2O_5 gave N, 7·94, 7·56, 7·37; P, 16·72, 16·83, 16·81 %. $C_3H_8O_6NP$ requires N, 7·57; P, 16·76 %.

Phosphoserine crystallizes in irregular platelets which darken on heating and melt at 165–166° with decomposition. A 0·5 % solution gave insoluble salts only with Pb acetate and $HgNO_3$.

4. *Phospho*iso*serine*

Phosphorylation of *iso*serine, prepared by Burch [1930], did not take place on standing at room temperature for 54 days. Poor yields (13–16 %) resulted on heating 1 g. *iso*serine, 7 g. H_3PO_4 and 1 g. P_2O_5 at 20 lb. pressure for 1–1·5 hr. The isolation was as described under phosphoserine.

The Ba salt could not be obtained pure and with the small amount available conversion into phospho*iso*serine could not be attempted.

5. *Phosphothreonine*

The mixture of stereoisomers of β-hydroxy-α-aminobutyric acid was prepared by the method of West & Carter [1937]. A yield of 89 % of the mercuric acetate compound of β-methoxybutyric acid was obtained, and from this a 92·5 % yield of β-methoxy-α-bromobutyric acid. 10 g. portions were converted into β-methoxy-α-aminobutyric acid by heating with conc. NH_4OH in a pressure bottle in boiling water for 1·5 hr. The yields were 60–63 %. Hydrolysis with HBr gave yields of 60–68 % of β-hydroxy-α-aminobutyric acids. The pure compound, recrystallized from water and alcohol, had N, 11·74 % (calc. 11·76 %) and M.P. 225–226° with decomposition, which corresponds with 228–229° given by Adkins & Reeve [1938] for *dl*-threonine, as compared with 252–253° for *dl*-*allo*threonine.

In comparison with serine and *iso*serine threonine is readily converted into phosphothreonine. 2 g. portions were heated with 10 g. H_3PO_4 and 2 g. P_2O_5 on a water bath for 5–20 hr. The yield of Ba salt averaged 46 %. The air-dried salt heated at 100° *in vacuo* over P_2O_5 gave N, 3·93; P, 8·94; Ba, 42·06; H_2O, 9·56 %. $C_4H_8O_6NPBa$ requires N, 4·19; P, 9·26; Ba, 41·07; $+2H_2O$ requires H_2O, 9·73 %.

The Pb salt was air-dried and heated at 100° *in vacuo* over P_2O_5 for analysis. (Found: N, 2·97; P, 7·22; Pb, 51·74; H_2O, 10·10 %. $C_4H_8O_6NPPb$ requires N, 3·46; P, 7·67; Pb, 51·24; $+3H_2O$ requires H_2O, 11·84 %.)

Phosphothreonine prepared from the Pb salt by adding 2 vol. alcohol and recrystallizing was dried at 100° *in vacuo* over P_2O_5. (Found: N, 6·37; P, 14·24; H_2O, 3·10 %. $C_4H_{10}O_6NP$ requires N, 7·04; P, 15·56 %. $C_4H_{10}O_6NP,H_2O$ requires N, 6·45; P, 14·27; H_2O, 8·34 %.)

More H_2O was driven off at 115°, but the substance turned brown and partially decomposed.

Phosphothreonine crystallizes from water and alcohol in small square plates, massing in cubes on slow crystallization, M.P. 169° with decomposition. A 0·5 % solution gave insoluble salts only with Pb acetate and $HgNO_3$.

6. *Phosphohydroxyaspartic acid*

Hydroxyaspartic acid, prepared by Burch [1930], could not be phosphorylated either at room temperature, at 100° or at 20 lb. pressure.

Hydrolysis of the esters

Phosphotyrosine was used itself. In the other cases solutions of the Na salts were prepared from the Ba salts by decomposition with the calculated quantity of Na_2SO_4 and filtration from $BaSO_4$.

A. HCl. About 100 ml. of solution were prepared. 1 ml. was taken for estimation of total P and 10 ml. portions for inorg. P by precipitation with NH_4Mg citrate. The NH_4MgPO_4 was filtered off, washed with NH_4OH, dissolved in 2*N* HNO_3 and the P estimated by the micro-method of Plimmer [1933]. The results were as follows:

1. *Phosphotyrosine*

By *N* HCl

0·8 g. in 105 ml. *N* HCl

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
48 ,,	0·31	3·2
5 hr. at 100°	4·79	50·0
21 ,,	8·32	86·8
28 ,,	8·55	89·2
48 ,,	8·49	88·6
72 ,,	9·41	98·2
Total P	9·58	

2. *Phosphohydroxyproline*

By *N* HCl

50 ml. Na salt + 50 ml. 2*N* HCl

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 100°	0	0
4 ,,	0·33	12·4
8 ,,	0·55	20·7
24 ,,	1·18	44·3
32 ,,	1·36	51·1
48 ,,	1·66	62·4
72 ,,	1·82	68·4
Total P	2·66	

3. *Phosphoserine*

By *N* HCl

50 ml. Na salt + 50 ml. 2*N* HCl

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
24 ,,	0	0
48 ,,	0	0
24 hr. at 100°	1·23	60·6
48 ,,	1·49	73·4
72 ,,	1·69	83·2
Total P	2·05	

4. *Phosphoisoserine*

By *N*/2 HCl

50 ml. Na salt + 50 ml. *N* HCl

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
42 ,,	0	0
8 hr. at 100°	1·02	44·0
24 ,,	1·95	84·0
48 ,,	1·96	84·5
72 ,,	2·00	86·2
Total P	2·32	

5. *Phosphothreonine*

By *N* HCl

50 ml. Na salt + 50 ml. 2*N* HCl

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
24 ,,	0	0
48 ,,	0	0
6 hr. at 100°	0·89	18·3
12 ,,	1·83	37·6
18 ,,	2·50	51·3
24 ,,	3·04	62·4
48 ,,	4·26	87·5
Total P	4·87	

At 37° there was no hydrolysis by *N* HCl, except of phosphotyrosine which amounted to 3·2 % in 48 hr. At 100° all the esters were hydrolysed fairly rapidly.

B. NaOH. The samples were acidified with HCl before precipitation with NH_4Mg citrate. Silica from glassware caused trouble in filtration and difficulty in washing. It may retain some unhydrolysed ester, which will be subsequently hydrolysed by HNO_3.

1. *Phosphotyrosine*

By *N* NaOH

1 g. in 50 ml. H_2O + 50 ml. 2*N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
48 ,,	0·03	0·4
5 hr. at 100°	0·07	1·0
24 ,,	0·25	3·7
48 ,,	0·28	4·2
72 ,,	0·35	5·2
Total P	6·70	

3. *Phosphoserine*

(1) By *N*/2 NaOH

50 ml. Na salt + 50 ml. *N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
6 ,,	0	0
25 ,,	0	0
24 hr. at 100°	0·90	50·9
48 ,,	1·24	70·0
Total P	1·77	

(2) By *N*/4 NaOH

50 ml. Na salt + 50 ml. *N*/2 NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 100°	0	0
4 ,,	0	0
8 ,,	0·07	3·7
24 ,,	1·23	65·4
48 ,,	1·24	66·0
72 ,,	1·20	64·9
Total P	1·88	

(3) By *N*/2 NaOH in Cu flask

50 ml. Na salt + 50 ml. *N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 100°	0	0
2 ,,	0·05	2·8
4 ,,	1·27	71·7
8 ,,	1·32	74·6
24 ,,	1·26	71·2
Total P	1·77	

2. *Phosphohydroxyproline*

(1) By *N* NaOH

52 ml. Na salt + 52 ml. 2*N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 100°	0	0
6 ,,	0	0
24 ,,	0·06	2·3
Total P	2·66	

The inorg. P seemed to be from adsorption on silica from glass vessel. Hydrolysis repeated in Cu flask.

(2) By *N*/2 NaOH

50 ml. phosphohydroxyproline + 50 ml. *N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 100°	0	0
3 ,,	0·06	1·8
6 ,,	0·03	0·9
24 ,,	0·34	10·1
48 ,,	0·07	2·1
72 ,,	1·13	33·7
120 ,,	1·27	37·9
Total P	3·35	

Hydrolysis apparently occurred after 24 hr. The low P figure at 48 hr. is probably an error.

4. *Phosphoisoserine*

By *N*/2 NaOH

50 ml. Na salt + 50 ml. *N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
40 ,,	0	0
24 hr. at 100°	0	0
48 ,,	0	0
72 ,,	0	0
Total P	2·43	

5. *Phosphothreonine*

By *N* NaOH

50 ml. Na salt + 50 ml. 2*N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
24 ,,	0	0
48 ,,	0	0
6 hr. at 100°	1·08	21·7
12 ,,	1·82	36·5
37 ,,	2·98	59·8
61 ,,	3·77	75·7
85 ,,	4·16	83·5
Total P	4·98	

All the esters are stable to NaOH at 37°. The stability contrasts with the instability of the phosphoric acid groups in caseinogen and caseo-phosphopeptone, which are completely hydrolysed by $N/4$ NaOH in 24 hr. At 100° there is only a small hydrolysis of phosphotyrosine by $2N$ NaOH in 72 hr.; this contrasts with the complete hydrolysis of phenyl phosphate [Plimmer & Burch, 1929]. Phosphohydroxyproline is only appreciably hydrolysed after 48 hr. Phosphoserine is hydrolysed to the extent of 60–70 % in 24 hr., but phospho*iso*serine is not hydrolysed. Phosphothreonine is hydrolysed like phosphoserine.

C. Kidney phosphatase. The enzyme solution was prepared by grinding minced rabbits' kidneys with sand and extracting with water + toluene for 24–48 hr. The extract was strained through muslin. Control experiments with boiled extract were made at the same time. Inorganic P was estimated after acidifying the solutions with HCl and precipitation with NH_4Mg citrate + NH_4OH. The procedure was then as before. The filtration of the solutions was very slow owing to precipitation of protein. The inorganic P of the control was deducted from that of the enzyme solution to give the change in P content due to hydrolysis.

1. *Phosphotyrosine*

25 ml. phosphotyrosine soln.
+ 40 ml. extract. *p*H 4

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0·32	6·0
24 ,,	2·59	49·1
48 ,,	3·42	64·9
96 ,,	3·62	68·7
Total P	5·27	

3. *Phosphoserine*

50 ml. Na salt + 50 ml. extract. *p*H 6·5

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
6 ,,	0·06	3·3
24 ,,	1·22	67·7
54 ,,	1·41	78·3
74 ,,	1·35	75·0
120 ,,	1·35	75·0
Total P	1·80	

5. *Phosphothreonine*

50 ml. Na salt + 50 ml. extract. *p*H 7

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
5 ,,	0·40	10·8
24 ,,	1·80	48·6
29 ,,	2·11	57·0
48 ,,	2·60	70·3
72 ,,	3·09	83·5
Total P	3·70	

2. *Phosphohydroxyproline*

50 ml. Na salt + 50 ml. extract. *p*H 5

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
4 ,,	0	0
7 ,,	0·08	2·9
23 ,,	0·82	29·7
30 ,,	1·00	36·2
48 ,,	1·57	56·9
72 ,,	2·10	76·1
96 ,,	2·07	75·0
Total P	2·76	

4. *Phospho*iso*serine*

50 ml. Na salt + 50 ml. extract. *p*H 7

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
8 ,,	8·5	45·2
24 ,,	10·2	54·2
48 ,,	11·6	61·1
72 ,,	10·8	57·4
96 ,,	11·1	59·0
Total P	18·8	

D. Intestinal phosphatase. The solution of enzyme was prepared from the mucous membranes of rabbits' intestines which were ground up with sand and extracted with water + toluene. Before use the extract was strained through muslin. The experiments were made as with kidney extracts. Filtration of the solutions was very slow owing to precipitation of proteins.

1. *Phosphotyrosine*

25 ml. phosphotyrosine soln. +40 ml. extract. pH 4

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	1·64	29·8
24 ,,	3·56	64·7
48 ,,	3·69	67·1
96 ,,	3·69	67·1
Total P	5·50	

2. *Phosphohydroxyproline*

50 ml. Na salt + 50 ml. extract. pH 6·5

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
3 ,,	1·05	59·0
7 ,,	1·14	64·0
22 ,,	1·23	69·1
46 ,,	1·18	66·3
72 ,,	1·37	77·0
96 ,,	1·30	73·0
Total P	1·78	

3. *Phosphoserine*

50 ml. Na salt + 50 ml. extract. pH 6·5

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
24 ,,	+	+
48 ,,	+	+
72 ,,	0·68	30·2
120 ,,	1·37	60·9
168 ,,	1·51	67·1
Total P	2·25	

+ error in determinations.

4. *Phosphoisoserine*

50 ml. Na salt + 50 ml. extract. pH 6·5

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
8 ,,	1·24	60·2
24 ,,	1·29	62·6
48 ,,	1·27	61·3
72 ,,	1·27	61·3
96 ,,	1·33	65·5
Total P	2·06	

5. *Phosphothreonine*

50 ml. Na salt + 50 ml. extract. pH 8

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
5 ,,	0·11	3·0
24 ,,	0·51	13·6
29 ,,	0·60	16·0
48 ,,	0·93	24·7
72 ,,	1·09	29·0
Total P	3·76	

Slow hydrolysis probably due to alkaline reaction.

All the esters are hydrolysed by kidney and intestinal phosphatases. The hydrolyses were never complete even after long periods of action. Comparison of the rates was not made as extracts made at different times were used.

SUMMARY

Phosphotyrosine, phosphohydroxyproline, phosphothreonine, phosphoserine and phospho*iso*serine have been prepared by phosphorylation of the amino-acids with $H_3PO_4 + P_2O_5$; in the case of phospho*iso*serine only the Ba salt was isolated. Phosphohydroxyaspartic acid could not be made in this way. The lead salts of all are insoluble in water, as also the mercurous salts except that of phosphohydroxyproline.

All the esters are hydrolysed by N HCl at 100°. All are stable to hydrolysis by $N/2$ or even N NaOH at 37°, but all except phospho*iso*serine are hydrolysed at 100°, phosphoserine and phosphothreonine fairly rapidly. All are hydrolysed by phosphatases of kidney and intestine.

REFERENCES

Adkins & Reeve (1938). *J. Amer. chem. Soc.* **60**, 1328.
Burch (1930). *J. chem. Soc.* p. 310.
Levene & Schormüller (1933). *J. biol. Chem.* **100**, 583.
—— —— (1934, 1). *J. biol. Chem.* **105**, 547.
—— —— (1934, 2). *J. biol. Chem.* **106**, 595.
Plimmer (1933). *Biochem. J.* **27**, 1810.
—— & Burch (1929). *J. chem. Soc.* p. 279.
—— —— (1937). *Biochem. J.* **31**, 398.
West & Carter (1937). *J. biol. Chem.* **119**, 103.

48. ON THE pH AT THE SURFACE OF OVALBUMIN MOLECULES, AND THE PROTEIN ERROR WITH INDICATORS

By JAMES FREDERIC DANIELLI[1]

From the Biochemical Laboratory, Cambridge

(*Received 12 March 1941*)

The pH in a surface phase is frequently different from that in a bulk phase in equilibrium with the surface. The surface pH may be calculated from the Gibbs-Donnan equilibrium [Danielli, 1937] using the equation

$$\frac{(\alpha_{Na})_s}{(\alpha_{Na})_b}=\frac{(\alpha_H)_s}{(\alpha_H)_b}, \tag{1}$$

where α denotes activity and suffixes s and b denote surface and bulk phases. If the various ionic species considered in any one phase all have the same activity coefficient, or if a given ion has the same activity coefficient in both phases, then the activity coefficients will cancel, giving

$$\frac{[Na]_s}{[Na]_b}=\frac{[H]_s}{[H]_b}, \tag{2}$$

where the square brackets refer to concentrations. Or, taking logarithms, and using the suffix D to denote this method of calculation,

$$(pH_s)_D-pH_b=-\log\frac{[Na]_s}{[Na]_b}. \tag{3}$$

If we had considered a diffusible anion instead of a cation, e.g. Cl^- instead of Na^+, we should have

$$(pH_s)_D-pH_b=+\log\frac{[Cl]_s}{[Cl]_b}. \tag{4}$$

Equation (3) or (4) can thus be taken as one method of defining the difference between pH_s and pH_b. Harvey & Danielli [1938] pointed out that these equations predicted that the pH at the surface of protein molecules is different from the bulk pH.

Linderstrøm-Lang [1924] pointed out that the concentration of ions at the surface of a colloidal particle is different from that in the surrounding bulk phases, and that the concentration ratio will be determined by the electrical potential between the surface of the colloid and the bulk phase. Linderstrøm-Lang predicted that the titration curves of proteins would be straight lines in the region of the iso-ionic point, and that the addition of salt would change the slope of these lines. This prediction was confirmed experimentally by Linderstrøm-Lang & Lund [1926]. But Linderstrøm-Lang did not calculate the actual pH at the surface of a colloidal particle.

Hartley & Roe [1940] have shown that pH_s may also be calculated from the electrokinetic potential ζ, using the equation

$$(pH_s)_E-pH_b=\frac{\zeta}{60}=0{\cdot}325u \tag{5}$$

for small particles at 25°. u is the mobility in μ/sec./volt/cm.

---

[1] Beit Memorial Medical Research Fellow.

Both methods of calculating $p\mathrm{H}_s$ give the average value of $p\mathrm{H}_s$ in the surface phase. Actually variation in $p\mathrm{H}_s$ must occur within the surface phase, as a function of distance from the charged groups of the surface. There is some theoretical difference between $(p\mathrm{H}_s)_D$ and $(p\mathrm{H}_s)_E$, since $(p\mathrm{H}_s)_D$ can, strictly speaking, give only the *activity* of the H^+ ion in the surface phase, and $(p\mathrm{H}_s)_E$ can only give the *concentration* of H^+ in the surface phase.

This paper deals with (1) the calculation of $(p\mathrm{H}_s)_D$ and $(p\mathrm{H}_s)_E$ at the surface of ovalbumin molecules, (2) the correction of titration curves for the difference between bulk and surface pH, (3) the nature of the 'protein' error with indicators.

(1) *The* p*H at the surface of ovalbumin molecules*

Fig. 1 shows results for ovalbumin. $[(p\mathrm{H}_s)_E - p\mathrm{H}_b]$ is plotted against $p\mathrm{H}_b$, using the mobility measurements of Abramson [1934]. On the acid side of the isoelectric point the pH at the surface of the protein is greater than in bulk and on the alkaline side is less than in bulk.

Calculation of $(p\mathrm{H}_s)_D$ is more complicated. We need to know the molar concentration of Na^+ or Cl^- in the surface phase of the protein. We can obtain an approximate value (to be corrected later) by assuming that, when the protein is ionized as an acid, no Cl^- enters the surface phase, and that when ionized as a base no Na^+ enters the surface phase. Then the number of ions in the surface phase of one protein molecule will be approximately equal to the valency z of the protein molecule. The valency can be obtained from the titration curve. The theory of Debye & Hückel enables us to calculate the thickness δ of the surface phase. Then the volume of the surface phase is $V = \frac{4}{3}\pi[(r+\delta)^3 - r^3]$, where r = radius of the protein molecule. For ovalbumin $r = 2\cdot75 \times 10^{-7}$ cm. [Adair & Adair, 1940] and in $N/100$ uni-univalent electrolyte $\delta = 3\cdot1 \times 10^{-7}$ cm. Hence $V = 7\cdot5 \times 10^{-19}$ ml. per molecule. Then the concentration of ions in the surface phase is

$$= \frac{z \times 1000}{V \times 6\cdot06 \times 10^{23}} = 2\cdot2z \times 10^{-3}\ \text{g. mol./l.} \tag{6}$$

This assumes that none of the mobile ions neutralizing the fixed charges on the protein surface can penetrate into the interior of the protein molecule. Having obtained this first approximation to the concentration in the surface, we can now use the Gibbs-Donnan equation:

$$\frac{[\mathrm{Na}]_s}{[\mathrm{Na}]_b} = \frac{[\mathrm{Cl}]_b}{[\mathrm{Cl}]_s} = \text{constant} \tag{7}$$

and the condition for electrical neutrality in the interface:

$$[\mathrm{Na}]_s + [\mathrm{Cl}]_s + [\mathrm{Pr}]_s = 0 \tag{8}$$

to calculate the true value of $[\mathrm{Na}]_s$ by successive approximations. $[\mathrm{Pr}]_s$ = concentration of ions due to groups fixed on the protein; e.g. for the case where $z = 10$, the protein is an anion and the bulk phase is $N/100$ NaCl, we have as a first approximation $[\mathrm{Na}]_s = 10 \times 2\cdot2 \times 10^{-3} = 0\cdot022$. Then from equation (7):

$$\frac{0\cdot022}{0\cdot01} = \frac{0\cdot01}{[\mathrm{Cl}]_s}$$

or

$$[\mathrm{Cl}]_s = 0\cdot0045.$$

Hence a better approximation to $[\mathrm{Na}]_s$ is $0\cdot022 + 0\cdot0045 = 0\cdot0265$. Then this value of $[\mathrm{Na}]_s$ can be substituted in (7) to get a more accurate value of $[\mathrm{Cl}]_s$. By a series of such successive approximations we obtain values for $[\mathrm{Cl}]_s$ and $[\mathrm{Na}]_s$ which are accurate. Having obtained $[\mathrm{Na}]_s$, and knowing $[\mathrm{Na}]_b$ and $p\mathrm{H}_b$, we can now calculate $(pH_s)_D$ from equation (3). The results of such calculations are shown in Fig. 1.

In the above calculation there are three sources of error, whose magnitude may be estimated. (1) The possibility of some of the mobile ions in the surface phase being inside the micelle has been neglected. Suppose that the mobile ions can penetrate readily into the whole of the protein molecule—this will be the greatest possible degree of penetration. Then we must regard as the volume in which the mobile ions are distributed not only the external layer, given by the Debye-Hückel theory, but also a layer inside the molecule of the same thickness, i.e. $3{\cdot}1 \times 10^{-7}$ cm. in $N/100$ solutions. This is greater than the radius of the ovalbumin molecule and so in practice we must simply add to the previously obtained value of V the volume of the ovalbumin molecule, obtaining $V' = 8{\cdot}37 \times 10^{-19}$ ml. per molecule instead of $7{\cdot}5 \times 10^{-19}$ ml. as the volume in which the z mobile ions are distributed. Using V' instead of V in equation (6) will lead to a reduction in $[(pH_s)_D - pH_b]$ of not more than 0·04 pH units. This is the maximum possible error introduced by ignoring penetration of ions into the interior of the ovalbumin molecule.

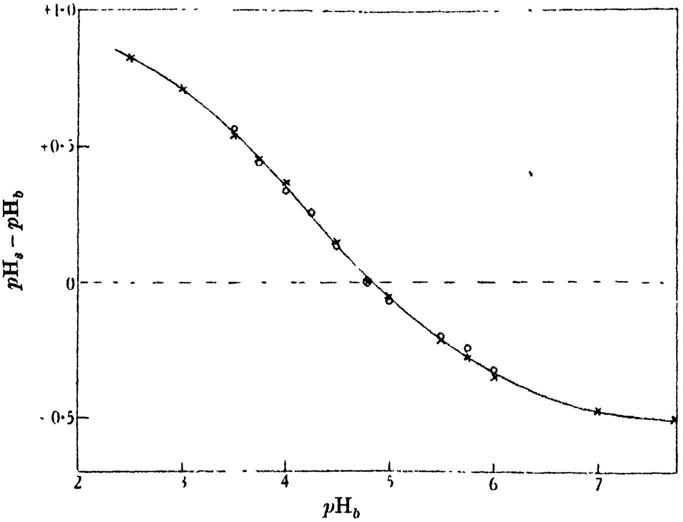


Fig. 1. Values of $(pH_s - pH_b)$ plotted against pH_b. × Calculated from Gibbs-Donnan equilibrium; o calculated from electrokinetic mobility.

(2) The values of z have been taken from the results of Kekwick & Cannan [1936] for salt-free solutions of ovalbumin. In 0·01 M NaCl this value may be up to 10 % too low [Cannan, 1938]. Hence the value of z used in equation (6) may be too low by this amount, and $(pH_s)_D - pH_b$ will be too low by not more than 0·04 pH unit. Errors (2) and (3) are of opposite sign.

(3) Adsorption of ions by processes other than those considered here, i.e. specific adsorption, would also introduce an error. The results of Adair & Adair [1940] suggest that this effect is quite small for ovalbumin.

From this discussion it follows that these errors may lead to the values of $[(pH_s)_D - pH_b]$ given in Fig. 1 being in error by a factor of not more than ±0·04 pH unit. We may therefore conclude that the values of the pH at the surface of ovalbumin molecules in 0·01 M NaCl solution, calculated by the two methods of equation (3) and equation (5), are in approximate agreement, since the points calculated by the two methods fall approximately in the same place.

(2) *The influence of ionic strength on surface* p*H and on the titration curve of ovalbumin*

We may write equation (4), for molecules on the acid side of the isoelectric point in the form

$$(p\mathrm{H}_s)_D = p\mathrm{H}_b + \log\,[\mathrm{Cl}]_s - [\mathrm{Cl}]_b. \tag{4a}$$

From this it follows that $p\mathrm{H}_s$ is a function of the salt concentration in the bulk phase. The actual degree of dissociation in the surface phase depends primarily on the $p\mathrm{H}_s$, and not on $p\mathrm{H}_b$: hence it follows that, at the same $p\mathrm{H}_b$, a protein will be dissociated (as an acid or base) to different degrees when different amounts of salts are present. In other words, the titration curves, that is, curves of acid or alkali combined with the protein plotted against $p\mathrm{H}_b$, will be different for different salt concentrations. If, however, we were to plot the amount of combined acid or alkali against $p\mathrm{H}_s$, we should eliminate the effect of variation in $p\mathrm{H}_s$ with salt concentration. Of course, $p\mathrm{H}_s$ cannot be determined directly, but it can be calculated by either of the methods given above.

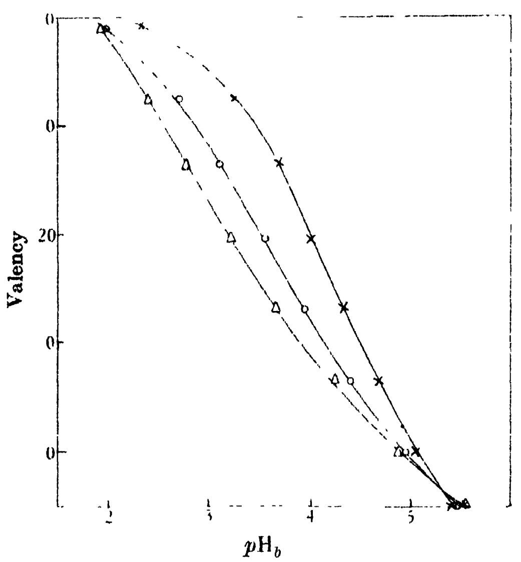


Fig. 2.

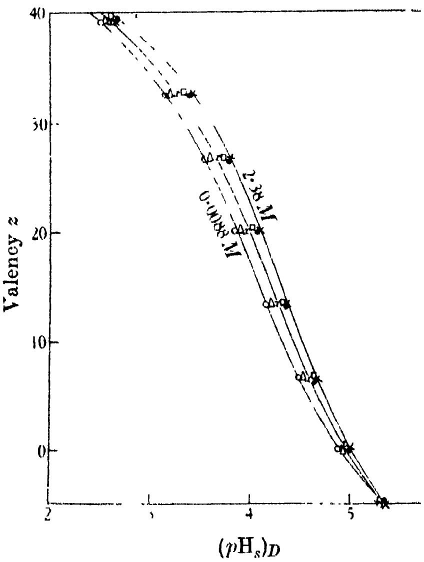


Fig. 3.

Fig. 2. Titration curves of ovalbumin in 2·38, 0·067 and 0·0088 M KCl solution. Valency z plotted against $p\mathrm{H}_b$. ×-- ×--× 2·38 M, △ - △- -△ 0·0088 M.

Fig. 3. Titration curves of ovalbumin in various dilutions of KCl. Valency z plotted against $p\mathrm{H}_s$. The 3 curves are for 2·38, 0·067 and 0·0088 M KCl. ×—×—× 2·38 M, ●—●—● 0·67 M, □—□—□ 0·133 M, ×—×—× 0·067 M, △--△—△ 0·033 M, ○—○—○ 0·0088 M.

Consider ovalbumin: Cannan [1938] has published titration curves for this protein in nine different concentrations of KCl. Fig. 2 shows his curves for 0·0088, 0·067 and 2·38 M KCl. The $p\mathrm{H}_b$ at which a given amount of acid is combined with the protein, i.e. at which the protein has a given valency z, varies by up to 0·9 pH unit between 0·0088 and 2·38 M KCl; as would be predicted from equation (4a), the more dilute the bulk phase, the more acid is the $p\mathrm{H}_b$ at which a given valency is achieved.

Fig. 3 shows the same data, plotted against $p\mathrm{H}_s$ as calculated from equations (3) and (4). The greater part of the variation with salt concentration is eliminated, and instead of the family of curves of varying form of Fig. 2, we get a series of curves which are substantially the one curve displaced along the $(pH_s)_D$ axis. The method by which $(pH_s)_D$ was calculated was as follows. (1) Approximate values for the surface concentration C_s of the ion of opposite sign to the protein were calculated for various dilutions of KCl. This involved calculation of (*a*) δ, the thickness of the double layer, (*b*) V, the volume of the double layer, and (*c*) the approximate value of C_s as the valency z multiplied by a constant typical of a given salt concentration. Table 1 shows this calculation. The next step (2) was to calculate the approximate values of C_s for a given z but different KCl dilutions from the values in Table 1, then correct this value of C_s by the method of successive approximations. Then (3) $(pH_s)_D$ was finally calculated from equation (3) or (4). A typical example of such calculations is shown in Table 2. Values of $\Delta pH = (pH_s)_D - pH_b$ are also given and will be observed to increase with increasing dilution of KCl. The individual values of $(pH_s)_D$ are subject to an error not greater than $\pm 0{\cdot}05$ pH unit, apart from any systematic error.

Table 1. *Calculation of the approximate concentration* C_s *in the surface phase of the ion of opposite sign of charge to that of ovalbumin*

C_s is given in the last column as a function of the valency z of ovalbumin. The radius r of ovalbumin is taken as $2{\cdot}75 \times 10^{-7}$ cm. $\delta = 3{\cdot}1 \times 10^{-8} - \sqrt{C_b}$.

	$\delta \times 10^7$	$(r+\delta) \times 10^7$	$(r+\delta)^3 \times 10^{21}$	$V \times 10^{20}$	$C_s \times 10^{-2}$ (1st approximation)
2·38	0·21	2·96	25·9	2·18	7·6z
1·19	0·28	3·03	28	3·06	5·4z
0·67	0·38	3·13	30·6	4·15	3·99z
0·27	0·60	3·35	37·5	7·05	2·35z
0·133	0·85	3·60	46·5	10·8	1·53z
0·067	1·20	3·95	61·5	17·1	9·7z
0·033	1·70	4·45	88	28·2	5·85z
0·017	2·38	5·13	134	47·5	3·49z
0·0088	3·3	6·05	222	84·5	1·95z

Table 2. *Calculation of* $(pH_s)_D$ *from the data of Table 1, and equations (7), (8) and (4a)*

Case of $z = 26{\cdot}3$.

C_b	C_s 1st approximation	C_s corrected	pH_b	$(pH_s)_D = pH_b - \log C_b + \log C_s$	$\Delta pH = (pH_s)_D - pH_b$
2·38	2·0	3·58	3·70	3·88	0·18
1·19	1·42	2·1	3·55	3·81	0·26
0·67	1·048	1·38	3·5	3·81	0·31
0·27	0·62	0·72	3·35	3·78	0·43
0·133	0·402	0·44	3·22	3·74	0·52
0·067	0·255	0·27	3·10	3·71	0·61
0·033	0·154	0·161	2·88	3·61	0·73
0·017	0·092	0·095	2·80	3·55	0·75
0·0088	0·051	0·053	2·75	3·53	0·78

The fact that, when z is plotted against $(pH_s)_D$ we get not one but several curves, shows that variation in ionic strength at constant pH_b has other effects in addition to affecting pH_s. There are probably many factors involved. The liquid junction potential involved in the measurement of pH_b varies slightly with ionic strength. The dissociation constants even of monocarboxylic acids vary slightly with change in ionic strength. In addition, it has been assumed in the above calculations that the surface area of ovalbumin molecules is independent of ionic strength: this may well not be true. Also the possibility that ions may enter the interior of the protein molecule has been neglected.

Hartley & Roe [1940] suggest that curves of z plotted against pH_s, rather than pH_b, should be used in determining the nature of the ionizing groups of proteins. It is certainly true that Fig. 3 presents a picture much more susceptible to analysis than is Fig. 2. If, as Cannan suggests, the titration curve of ovalbumin between *p*H 2 and *p*H 7 is that of a polycarboxylic acid, the apparent *p*K of this acid, from Fig. 3, lies between 4·15 and 4·4.

To carry out the suggestion of Hartley & Roe involves calculating pH_s either from the electrokinetic mobility or from the Gibbs-Donnan equilibrium, using the data of the titration curve itself, as has been done above for ovalbumin. Systematic studies of variation in mobility with wide variation in *p*H and ionic strength have not yet been made, so that the method of Hartley & Roe is not always available. On the other hand, calculation from the Gibbs-Donnan equilibrium involves knowledge of the shape and surface area of the protein concerned—this again is seldom available. A possible solution of this difficulty is to obtain values of the electrokinetic mobility at several *p*H, and from these to calculate the charge density. Then, since the titration curve gives the valency z, i.e. total charge per molecule, multiplying the charge density by z will give the surface area, which may then be used for other *p*H values. This suggestion, however, needs very careful experimental checking.

(3) *The 'protein error' in* pH *determinations with indicators*

Many workers, e.g. Sörensen [1909], have studied the effect of proteins on colorimetric *p*H determinations. As a general rule, where the indicator and protein have opposite charges, the protein error may be large. Where the indicator and protein have the same sign of charge, the protein error is usually small.

Danielli [1937] pointed out that much of the error in *p*H determinations with indicators is due to adsorption of indicators at surfaces whose *p*H is not that of the bulk phase. The indicator, if adsorbed, tends to give the *p*H of the surface phase, not of the bulk phase. Hartley [1934] has made a wide study of the indicator error with solutions containing paraffin chain acids and bases, finding that with solutions containing micelles of basic substances (whose surfaces are more alkaline than the bulk phase) the *p*H given by the indicator is displaced from the bulk value towards the alkaline side, whereas with micelles of acidic substances the displacement is in the reverse direction. A most striking effect is that where warming of the solution causes dissolution of the micelles; as the dissolution becomes complete, the indicator colour changes sharply to that characteristic of the bulk *p*H. This change may be as large as two *p*H units.

A large part of the protein error with indicators is probably due to this effect. Most of the evidence in the literature is fragmentary, but it is true of these scattered observations that with indicators which are anions, the indicator error is large and positive on the acid side of the isoelectric point of a protein and small on the alkaline side. With indicators which are cations the relationships are reversed. This is the type of variation to be expected if the indicator error is mainly due to adsorption of indicator at the protein surface: if a protein is positively charged it will tend to adsorb large organic anions but not cations, and since the *p*H at the protein surface is greater than in bulk, the 'protein error' is positive. These relationships are reversed at a negatively charged surface. St Johnston & Peard [1926] have made a study of the sulphonethalein indicators with gelatin: their results are in agreement with the above theoretical conclusions, but, as the colour comparison was made by eye, their individual results are in error by 0·05–0·1 *p*H unit, so that the points are scattered rather widely.

Fig. 4 shows some more accurate determinations on 1 % ovalbumin solutions, made with a photoelectric colorimeter, which allows determinations to be made to ±0·02 *p*H unit on standard buffer solutions. The bulk *p*H values were measured with a glass electrode. Three indicators were used: bromocresol green and chlorophenol red being anionic, and neutral red cationic. Fig. 4 shows that with the anionic bromocresol green the protein error is positive on the acid side of the isoelectric point and almost zero on the alkaline side. With chlorophenol red the $p\mathrm{H}_b$ values all lay on the alkaline side and the protein error was small. But with neutral red, with which again the $p\mathrm{H}_b$ values were all on the alkaline side, the protein error was large and negative. Qualitatively these results are in agreement with theoretical predictions.

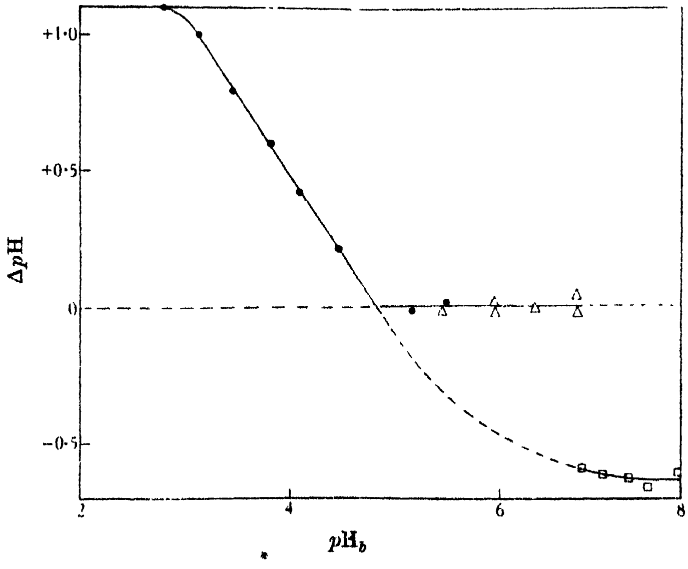


Fig. 4. The protein error, Δ*p*H, for ovalbumin solutions in 0·01*M* NaCl, plotted against $p\mathrm{H}_b$. ● Bromocresol green; △ chlorophenol red; □ neutral red.

The ovalbumin was four times recrystallized, dialysed salt-free and diluted to a 4 % solution. NaCl was added to bring the salt concentration to 0·01*N*. 5 ml. samples of the solution were titrated with 0·01*N* NaOH or HCl in 0·01*N* NaCl to suitable $p\mathrm{H}_b$ values, with a standard amount of indicator present, and then diluted to 20 ml. with 0·01*N* NaCl: the final solution contained 1 % ovalbumin. The *p*H values of these solutions were measured first with a photoelectric colorimeter, then with the glass electrode, both determinations being made on the same solution, the difference between the two values being the protein error. The colorimeter was calibrated by using solutions of measured *p*H containing 0·01*N* NaCl and 0·0002*M* acetate buffers and the same standard amount of indicator.

Discussion

The previous examination of the results for ovalbumin shows that the *p*H at the surface of ovalbumin molecules may be quite different from that in bulk solution and that it is affected by salt concentration as well as by bulk *p*H. In the case of colloidal enzymes, as was suggested to me by Prof. J.C. Drummond in 1935, this may be a matter of considerable significance. In 0·01 N solutions, the thickness of the ionic double layer is about 30 Å.: this is considerably greater than the diameter of many substrate molecules, so that in such a solution the enzyme-substrate interaction must occur within the double layer, and the significant pH determining the rate of reaction is probably the surface pH. Varying the ionic strength of the medium at constant pH_b must vary pH_s, and it seems probable that the greater part of the effect of neutral salts on enzymes is due to this variation in pH_s. Whether this is actually so must await more systematic studies than are yet available. It is, of course, not practicable in general practice to measure pH_s in most cases. But if the ionic strength of the substrate is kept constant when the pH is changed a standard set of conditions will result, so that one observer can compare his results exactly with those of another; e.g. if two sets of results are obtained with an ionic strength of 0·1, they may be compared exactly, for the same colloid in both sets of experiments will have the same pH_s at a given pH_b, even though the pH_s values cannot be measured. The simplest procedure is probably to carry out all experiments in say 0·5 or 0·1 N NaCl, and not to allow the molar concentration of buffer substances to rise above 1 % of the NaCl; under these conditions the ionic strength will be practically constant. Polyvalent ions are to be avoided as far as possible.

Both methods of calculating pH_s determine the average pH in the surface phase, assuming that the charges are evenly distributed over the colloid surface and it is assumed that pH_s is the same throughout the surface phase. It is quite possible that this is not true and that there is a considerable local variation in pH_s. Neither method of calculation can detect such local variations. It is possible, however, that the results with indicators are partly due to local variations. The protein error ΔpH of Fig. 4 is roughly 1·4 ($pH_s - pH_b$) of Fig. 1, for indicators of opposite charge to the protein; i.e. although qualitatively the protein error follows the difference between the surface and bulk pH values, quantitatively it is 40 % larger than can be due to the calculated values of pH_s, even if the whole of the indicator were adsorbed. This factor of 1·4 may be due to the ionizing groups being concentrated in patches on the protein surface. But there are many other possible explanations and no conclusion can be reached until the distribution of other ions is studied.

The views advanced here are not intended to constitute a complete theory of the protein error with indicators. Many other factors, such as the different valencies of the two forms of an indicator, and molecular interactions such as have been recently studied by Schulman & Rideal [1937], must also be involved.

SUMMARY

1. pH_s, the pH at the surface of a protein molecule, is different from that in the surrounding bulk phase. pH_s may be calculated either from the Gibbs-Donnan equilibrium, or from the electrokinetic mobility. For ovalbumin the two methods give results in reasonable agreement.

2. At constant bulk pH, pH_s varies with ionic strength, and consequently the acid- and base-binding powers of a protein vary with ionic strength. It is shown that this accounts for the major part of the influence of ionic strength on the titration curve of ovalbumin, between pH 2 and pH 5·5.

3. Indicators of opposite charge to a protein molecule tend to be adsorbed and give values of pH_s rather than bulk pH values. Results are given for ovalbumin solutions with bromocresol green, chlorophenol red and neutral red. It is

not clear whether the whole of the protein error is due to the difference between pH_s and the bulk pH.

4. It is suggested that pH_s, and not pH_b, is the significant pH in enzyme activity, and that, to facilitate comparison of results, enzyme activities should be determined in solutions of constant ionic strength.

I am deeply indebted to Dr G. S. Adair, Dr A. Neuberger and Dr J. H. Schulman for reading the manuscript and for a number of valuable suggestions.

REFERENCES

Abramson (1934). Electrokinetic Phenomena. New York.
Adair & Adair (1940). *Trans. Faraday Soc.* **36**, 26.
Cannan (1938). *Cold Spring Harbour Symposia*, **6**, 1.
Danielli (1937). *Proc. roy. Soc.* B, **122**, 155.
Hartley (1934). *Trans. Faraday Soc.* **30**, 444.
—— & Roe (1940). *Trans. Faraday Soc.* **36**, 105.
Harvey & Danielli (1938). *Biol. Rev.* **13**, 319.
Kekwick & Cannan (1936). *Biochem. J.* **30**, 227.
Linderstrøm-Lang (1924). *C.r. Carlsbad*, **15** (7).
—— & Lund (1926). *C.r. Carlsbad*, **16** (5).
Schulman & Rideal (1937). *Proc. roy. Soc.* B, **122**, 29, 46.
St Johnston & Peard (1926). *Biochem. J.* **20**, 817.
Sörensen (1909). *Biochem. Z.* **21**, 255.

49. THE METABOLISM OF RAT LIVER DURING CARCINOGENESIS BY BUTTER YELLOW

By JOHN WASHINGTON ORR and LEONARD HUBERT STICKLAND

From the Department of Experimental Pathology and
Cancer Research, University of Leeds

(*Received 7 March 1941*)

The production of liver tumours in rats by the inclusion of azo-dyes in their diet was first demonstrated by Yoshida [1932; Sasaki & Yoshida, 1935]. In this work the dye used was *o*-aminoazotoluene (2:1:1:4:3-tolueneazoaminotoluene). Later butter yellow (dimethyl yellow; *p*-dimethylaminoazobenzene) was found to produce the same result more quickly and with a higher yield of tumours [Kinosita, 1937]. The whole subject has been thoroughly reviewed by Kinosita [1937].

These tumours were shown [Nakatani *et al.* 1938] to possess the type of metabolism which is, according to the majority of observers, characteristic of tumours in general, viz. a high rate of anaerobic glycolysis and a considerable rate of aerobic glycolysis. Normal rat liver, on the other hand, according to Warburg *et al.* [1924] shows a low anaerobic glycolysis ($Q_{CO_2}^{N_2}$ ~4) and negligible aerobic glycolysis ($Q_{CO_2}^{O_2}$ ~1). The Japanese workers further found that during the period preceding the appearance of a tumour the liver showed a progressively increasing anaerobic glycolysis, from the normal value of $Q_{CO_2}^{N_2}$ ~4 up to $Q_{CO_2}^{N_2}$ ~12. The aerobic glycolysis meanwhile remained at its normal low level. When tumours appeared, they showed the type of metabolism usually associated with tumours, while the remainder of a tumour-bearing liver retained its previous metabolism (high anaerobic, low aerobic glycolysis).

In our first experiments on this subject we were unable to obtain consistently the low values of $Q_{CO_2}^{N_2}$ in normal liver which are quoted by Warburg *et al.* [1924] and Nakatani *et al.* [1938]. Our values ranged indiscriminately between 3 and 15. On consulting the literature, we found that we and the Japanese workers had overlooked the work of Rosenthal [Rosenthal & Lasnitzki, 1928; Rosenthal, 1929], which fundamentally changes the nature of the problem. Rosenthal's results would seem to suggest that (1) liver is not able to glycolyse glucose added to the external medium, but only the glycogen contained in the cells, (2) the rate of this autoglycolysis depends on the state of nutrition of the rat, i.e. presumably on the concentration of glycogen in the liver. In starved animals $Q_{CO_2}^{N_2}$ may be as low as 1, in well-fed ones as high as 16.[1]

---

[1] Rosenthal found that liver slices placed directly in N_2 showed only a small glycolysis, the high values in well-fed rats being shown by slices removed to N_2 after preliminary incubation in O_2. 30 min. incubation in O_2 at either 20° or 37° sufficed to produce the maximum value of $Q_{CO_2}^{N_2}$. We have failed to observe this phenomenon, and our results under conditions of 'direct' anaerobiosis obviously correspond with his after a preliminary aerobiosis. Perhaps there was a greater delay in our case (up to 20 min. from the killing of the rat to the establishment of anaerobiosis), and our slices had in effect been incubated in air at 20° for a sufficient period to raise $Q_{CO_2}^{N_2}$ to its maximum value.

These considerations make the interpretation of the results of Nakatani *et al.* [1938] very difficult, and we decided to study the following points:

(1) The highest value of $Q_{CO_2}^{N_2}$ recorded by Nakatani *et al.* for liver slices before the appearance of a tumour lies well within our normal range. Is there any evidence of an increase of anaerobic glycolysis of the liver during butter yellow feeding?

(2) Normal liver does not glucolyse. Does butter yellow feeding cause the liver to acquire the power of glucolysis at any stage before the appearance of a tumour?

(3) The glycolytic mechanism of all other tumours so far investigated acts only on monosaccharides. Is this also true of the tumours produced in liver?

(4) There are two main types of carcinomata produced in liver by the action of butter yellow, one derived from the liver parenchyma (hepatomata), and the other from bile-duct epithelium (cholangiomata). In normal liver the liver cells predominate to such an extent that the values for the metabolism of the tissue may be taken to represent the metabolism of the liver cells only. The metabolism of normal bile-duct epithelium is therefore unknown. Do liver cells, when they form tumours, show an abrupt change in metabolism from glycogen glycolysis to glucolysis?

This paper provides an answer to the first three of these questions. On account of the breaking off of our experiments in September 1939, the answer to the fourth remains for the present only tentative.[1]

EXPERIMENTAL

Administration of butter yellow to rats. White stock rats, obtained from a dealer, were used. At the start of the experiment they were almost, but not quite, full-grown. The first group were fed on a mixture of wheat, maize and oats, with supplements of green vegetables and water *ad lib.*; in a later group the cereal food was unpolished Indian rice, as there is evidence [Ando, 1938] that the changes develop more rapidly in rice-fed than in wheat-fed animals. There was no significant difference in the yield or rate of appearance of tumours, or in the findings now reported, as between these two experiments.

The dye used was 'dimethyl yellow (Analar)' and was obtained from British Drug Houses. It was dissolved in warm olive oil in a concentration of 3 %; on cooling, a very small amount crystallized out, and the saturated solution was added to, and intimately mixed with, the cereal part of the animals' food in the proportion of 20 or 30 ml./kg.

It is important to point out that the changes in the livers of our animals progressed at a much slower rate than has been described by the Japanese workers. The detailed histology has been fully described elsewhere [Orr, 1940]. Briefly, the earliest changes in the liver parenchyma are of a degenerative type. After a month, microscopical examination shows proliferation of connective tissue in the neighbourhood of the portal tracts. Later there is added to this regenerative proliferation of both liver tissue and bile ducts, in varying proportions. After about 3 months, as a result of all these processes, the liver has a granular appearance similar to that of ordinary cirrhosis, and histologically shows marked replacement of the normal lobular architecture by regeneration nodules of parenchyma and trabeculae of granulation tissue; this stage may conveniently be termed 'nodular hyperplasia'. Carcinomata were first found in the 5th month of treatment, the earliest ones being derived from bile duct epithelium (the so-called cholangiomata); tumours of liver cells proper (hepatomata) appeared rather later, but frequently both types of carcinoma were found in the same liver. It was therefore important that the actual tumour whose metabolism was measured should also be subjected to histological examination; this was done in all instances.

---

[1] An attempt since then to amplify our observations has been unsuccessful as a result of the failure of the rats to develop tumours after up to 15 months of butter yellow feeding. The principal difference has been that these rats have been fed on ordinary white bread with greenstuff supplements. In the original experiments the main diet was unpolished rice, or a mixture of wheat, maize and oats. We have not yet found it possible, under present circumstances, to investigate this problem.

Measurement of metabolism. The manometric methods employed were those described by Berenblum *et al.* [1936], but tissue slices were always used. It must be noted that this method of measuring the aerobic glycolysis, unlike that of Warburg [1924], gives only the glucolysis; both anaerobic methods of course measure the whole lactic acid production from whatever carbohydrate substrates may be present. For the determination of the rate of aerobic glycolysis in normal liver, therefore, the chemical method of lactic acid estimation has been used [Friedemann *et al.* 1927]. Manometric and chemical determinations in the same (anaerobic) experiment showed satisfactory agreement, the manometric figures being usually some 10 % higher than the chemical (cf. Table 7).

Estimation of glycogen concentration in liver. The usual method was used, the details being according to Hynd & Rotter [1930].

1. $Q^{N_2}_{CO_2}$ *of normal liver tissue*

As a result of our earliest experiments and consideration of Rosenthal's work, we decided that the estimation of liver glycolysis, without simultaneous

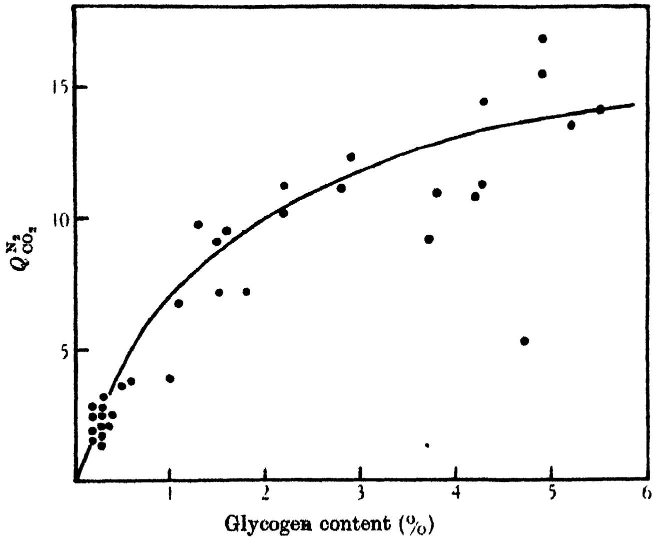


Fig. 1. Rate of glycolysis and glycogen content of normal livers.

determination of the glycogen content of the tissue, yielded results the interpretation of which was necessarily obscure. A number of rats with different liver glycogen concentrations was therefore used to establish the relationship between rate of glycolysis and glycogen concentration. Attempts were made to produce different glycogen contents by injection of varying amounts of glucose solution into starved rats at appropriate times before killing the animals, but such methods were found to be unreliable. Eventually sufficient liver glycogen values in the range from 1 to 5 % were obtained by random sampling from fed rats, and values from 0·1 to 0·5 % from rats starved for periods up to 48 hr.

The results are shown in Fig. 1. The points are rather scattered, but there is obviously an association between rate of glycolysis and glycogen concentration. It is seen that normal rat liver frequently shows a $Q^{N_2}_{CO_2}$ value of 15 or more, while the highest figure recorded by Nakatani *et al.* [1938] for the liver of rats fed on butter yellow was 11.

It is evident that a comparison of the average values of $Q^{N_2}_{CO_2}$ for a large number of rats of different liver glycogen levels is not a good criterion for determining whether butter yellow feeding induces any change in the rate of glycolysis. In practice, a fairly satisfactory comparison can be made by considering only those cases in which the liver glycogen was 1·5 % or greater. It can be seen from Fig. 1 that from 1·5 to 5 % glycogen the rate of glycolysis increases relatively slowly. Such data for normal livers are shown in Table 1.

Table 1. *Normal liver slices.* $Q^{N_2}_{CO_2}$ *and glycogen concentration*

$Q^{N_2}_{CO_2}$	Glycogen %	$Q^{N_2}_{CO_2}$	Glycogen %	$Q^{N_2}_{CO_2}$	Glycogen %
16·8	4·9	11·3	2·2	9·4	1·6
15·4	4·9	11·3	4·3	9·1	3·7
14·4	4·3	11·0	3·8	9·1	1·5
14·1	5·5	11·1	2·8	7·3	1·8
13·5	5·2	10·8	4·2	7·1	1·5
12·3	2·9	10·2	2·2	5·2	4·7
			Average	11·1	3·4

II. $Q^{N_2}_{CO_2}$ *of liver tissue from rats receiving butter yellow*

Nakatani *et al.* [1938] related the increase in rate of glycolysis of liver slices solely with the duration of butter yellow feeding. We find that the response to the butter yellow is very irregular; for instance, after some 3 months of feeding, some livers are macroscopically almost normal while others show advanced cirrhosis. In Table 2 we have therefore classified the livers roughly on the basis of the histological results (*v. supra*), under the following headings:

(*a*) Normal.

(*b*) Degenerative changes only.

(*c*) Periportal connective tissue proliferation without macroscopical granularity.

(*d*) Nodular hyperplasia with loss of architecture.

There is clearly no evidence here of a progressive increase in the rate of glycolysis parallel with the changes produced by the feeding of butter yellow.

In Table 3 we have analysed the data for the 20 livers of Table 2 (*c* and *d*), grouping them according to their glycogen contents, and comparing their glycolytic rates with those of a similar series of 18 livers from normal rats. It will be seen that while in both series there is the expected rise in glycolysis as glycogen content increases, in no case is the glycolysis of the butter yellow livers significantly higher than that of the corresponding normal groups. All these differences fall far below the conventional level of significance ($D/\text{S.E.} = 2$).

Table 2. $Q^{\mathrm{N}_2}_{\mathrm{CO}_2}$ *of livers from rats fed with butter yellow*

$Q^{\mathrm{N}_2}_{\mathrm{CO}_2}$	Glycogen %	$Q^{\mathrm{N}_2}_{\mathrm{CO}_2}$	Glycogen %	$Q^{\mathrm{N}_2}_{\mathrm{CO}_2}$	Glycogen %
(*a*) Livers which after various periods of butter-yellow feeding were histologically normal:					
14·1	5·5	11·1	2·8	9·1	1·5
13·5	5·2	9·8	3·8		
			Average	11·5	3·7
(*b*) Livers showing degenerative changes:					
11·6	3·0	11·0	3·6	6·4	5·0
			Average	9·7	3·9
(*c*) Livers showing periportal changes:					
14·4	4·0	8·7	2·6	6·7	1·9
14·3	4·1	7·9	3·7	6·4	3·7
13·2	5·5	7·7	3·2	4·5	2·8
12·9	3·0	7·5	2·0	3·7	2·1
11·8	3·1				
			Average	9·2	3·3
(*d*) Livers showing nodular hyperplasia:					
16·6	2·6	13·2	5·1	12·8	4·4
14·2	5·3	13·0	4·2	11·0	4·7
13·5	1·7				
			Average	13·5	4·0

Table 3. *Comparison of precancerous livers of butter yellow-fed rats with normal livers of similar glycogen content*

Range of glycogen %	Average $Q^{\mathrm{N}_2}_{\mathrm{CO}_2}$ ± standard error		
	Normal	Butter yellow	*D*/S.E.
1·5–2·5	9·1 ± 0·5	7·8 ± 2·1	−0·6
2·5–3·5	11·7 ± 0·6	10·4 ± 1·7	−0·7
3·5–4·5	11·3 ± 0·9	11·5 ± 1·4	+0·1
4·5–5·5	13·4 ± 2·0	12·9 ± 0·7	−0·2

III. *Glycolysis in regenerating liver*

Regeneration of surviving parenchyma plays an important part in the alteration of liver structure during cirrhosis, so for comparison we have made some measurements on regenerating livers. After removal of about one-half of the organ, a rat's liver regains its normal weight in about 8 days, and the maximal rate of growth, as estimated by the number of cells in mitotic division, occurs at about the 4th day. A number of livers were therefore studied on the 3rd, 4th or 5th day after the removal of one-half of the liver. The results are given in Table 4, and it is plain the $Q^{\mathrm{N}_2}_{\mathrm{CO}_2}$ of regenerating liver does not appreciably differ from that of normal liver.

Table 4. *Glycolysis of regenerating livers*

$Q^{\mathrm{N}_2}_{\mathrm{CO}_2}$	Glycogen %	$Q^{\mathrm{N}_2}_{\mathrm{CO}_2}$	Glycogen %	$Q^{\mathrm{N}_2}_{\mathrm{CO}_2}$	Glycogen %
16·7	4·96	14·9	6·20	13·4	3·32
6·7	2·40	15·1	3·18	14·4	3·95
14·7	4·13	12·3	4·30		
			Average	13·5	3·55

IV. *Absence of glucolysis in liver tissue*

In our series of measurements on normal liver there was apparent in general a slightly greater glycolytic rate in the presence of glucose than in its absence, and this difference was not appreciably altered by butter yellow feeding (Table 5). At the same time, we do not believe that this finding is in disagreement with the observations of Rosenthal & Lasnitzki [1928] and Dickens & Greville [1932] that normal liver does not glucolyse, because the differences are small, not invariable, and can be interpreted in more than one way (*v. infra*). The results of Rosenthal [1929] are in agreement with ours in showing a slight increase of $Q^{N_2}_{CO_2}$ in presence of glucose, though he makes no comment on this fact. A curious point is that rat liver readily attacks fructose, and that fructolysis is slight in starved animals and considerable in fed ones [Rosenthal, 1930; Dickens & Greville, 1932].

Table 5. *Glucolysis in liver tissue*

$Q^{N_2}_{CO_2}$		$Q^{N_2}_{CO_2}$		$Q^{N_2}_{CO_2}$	
Glucose absent	Glucose present	Glucose absent	Glucose present	Glucose absent	Glucose present
		(*a*) Normal and regenerating:			
2·1	2·8	2·2	2·8	5·8	7·4
0·5	1·3	1·2	1·7	3·7	5·0
9·3	8·0	8·2	7·1	12·3	12·0
4·8	5·5	6·7	7·0	15·1	17·6
16·7	17·6	5·8	7·1	2·7	3·5
3·0	3·7	1·8	2·4	3·1	3·2
2·3	3·1	0·8	2·3	14·1	12·7
13·5	16·2	9·7	8·4	11·1	11·1
9·1	6·9	9·3	8·0	8·2	7·1
			Average	6·8	7·1
		(*b*) Degenerative changes:			
11·0	12·7	11·6	11·3	6·4	9·4
			Average	9·7	11·1
		(*c*) Periportal changes:			
14·3	14·5	7·7	9·4	7·9	8·7
14·4	14·2	6·7	4·8	8·7	8·5
6·4	5·2	3·7	7·7	13·2	17·0
4·5	5·2	7·5	14·8	12·9	13·1
11·8	14·3	1·8	4·5	3·0	3·4
			Average	8·5	9·7
		(*d*) Nodular hyperplasia:			
13·0	16·9	12·8	16·6	13·2	14·2
11·0	13·2	13·5	15·2	1·9	4·6
2·0	4·4	14·2	15·4	3·1	7·3
16·6	12·2	4·5	7·7		
			Average	9·6	11·6

It is clear from these figures that there is no striking appearance of glucolysis during the feeding of butter yellow to rats, indeed it would require a very large number of observations to support a claim that there was any significant amount of glucolysis at all.

The same result is found with regenerating livers (Table 6).

Aerobically also liver slices glycolyse only glycogen. A large number of manometric measurements of aerobic glucolysis gave for $Q^{O_2}_{CO_2}$ an average figure

Table 6. *Glucolysis in regenerating livers*

	$Q^{N_2}_{CO_2}$	
	Glucose absent	Glucose present
	16·7	17·6
	5·8	7·1
	15·1	17·6
	4·8	5·5
	6·7	7·0
Average	9·8	11·0

of 1·1 ± 1·1, which is not significantly different from zero, while chemical estimation of the total aerobic glycolysis (lactic acid formation) gave the results in Table 7 (the anaerobic results for the same livers are given for comparison, simultaneous chemical and manometric estimations being given side by side to show the degree of agreement).

Table 7. *Glycolysis of normal liver slices*

The paired values in each column are derived from duplicate experiments with material from the same source.

$Q^{N_2}_{CO_2}$ (manometric)		$Q^{N_2}_{CO_2}$ (chemical)		$Q^{O_2}_{CO_2}$ (chemical)	
8·5	9·3	8·7	10·0	1·9	1·8
4·7	4·6	5·6	5·2	1·9	1·7
10·2	10·7	9·4	10·0	2·2	2·4
4·3	4·6	4·3	4·5	1·6	1·5
4·5	4·5	5·3	4·4	1·8	1·9
2·9	3·4	2·4	2·8	1·5	1·3
10·5	10·8	10·4	10·7	1·9	—
8·3	9·5	8·1	9·7	1·9	1·9

V. *The metabolism of the tumours induced by butter yellow*

Owing to the premature end of our experiments, our data on the metabolism of butter yellow tumours are scanty. They are collected in Table 8.

Table 8. *Metabolism of liver tumours*

	$Q^{N_2}_{CO_2}$		$Q^{O_2}_{CO_2}$
Type of tumour	Glucose absent	Glucose present	(glucolysis only)
Cholangioma	—	5·5	2·5
,,	—	13·2	5·2
,,	—	13·0	5·4
Hepatoma	—	20·8	8·6
,,	0·0	17·0	6·0
,,	1·9*	8·0*	—
,,	—	11·4	3·2

* This was not a single discrete tumour, but a number of separate nodules. The slices were cut so as to include as much tumour tissue as possible, but they were contaminated with some liver tissue.

Only two results are available which show directly the presence of anaerobic glucolysis in hepatomata. However, in the cases of the three cholangiomata and of the other two hepatomata there is a considerable aerobic glycolysis. As was stated before, our method of measuring aerobic glycolysis manometrically excludes the lactic acid production from any substrate except the added glucose, so these results all support the view that glucolysis does occur, both in cholangiomata and in hepatomata.

Discussion

From our results we may attempt to answer the four questions mentioned in the introduction (p. 480):

(1) There is no evidence of an increase in the rate of anaerobic glycolysis of liver tissue of rats at any stage of feeding of butter yellow. The increase found by Nakatani *et al.* [1938], unless there is a difference in behaviour between the strains of rats used by them and by us, must have been due to chance. They found an increase of $Q^{N_2}_{CO_2}$ from 4 in normal livers to 12 in cirrhotic livers, while we find that $Q^{N_2}_{CO_2}$ may lie anywhere between 2 and 16 in any rat, according to the glycogen concentration in the liver.

(2) Liver tissue does not acquire an increased power of glucolysis at any stage, prior to actual neoplasm, of butter yellow feeding. The presence of glucose causes an average increase of about 1 in the value of $Q^{N_2}_{CO_2}$, which increase is independent of the absolute value of $Q^{N_2}_{CO_2}$, i.e. is independent of the glycogen content. Among possible explanations of this are (*a*) that liver cells have a slight power of glucolysis, (*b*) that a small proportion of the cells of the liver (e.g. possibly the bile-duct epithelium as distinct from the liver cells) are glucolytic, or (*c*) that the presence of glucose increases the rate of glycogen breakdown.

(3) All the liver tumours we have studied possessed the power of glucolysis. In the two cases in which the point was specially studied, it was found that there was no autoglycolysis, i.e. no glycolytic breakdown of glycogen, and only glucose was attacked. However, histologically the tumour tissue fixed in alcohol was free from glycogen, so there is no possibility of testing whether the tumours would attack glycogen if that substrate were present.

(4) The answer to this important question depends on the results of only four experiments, so we give it only tentatively, and hope to obtain further data in confirmation later. It appears probable, however, that in the transition to malignancy there occurs abruptly a complete change in the type of metabolism of the liver cells, viz. the sole substrate used for glycolysis changes from glycogen to glucose.

Warburg was the first to show a qualitative difference in metabolism between normal and tumour tissues, his results showing that tumour metabolism was characterized by a high rate of glycolysis which was only partially suppressed by oxygen. Since then various normal tissues have been shown to have a similar type of metabolism, e.g. testis (rat), retina (rat) and hyperplastic tonsil (man) [Warburg *et al.* 1924], embryonic lens of rat [Fujita, 1928], kidney medulla in several species [György *et al.* 1928; Dickens & Weil-Malherbe, 1936] and bone marrow of rabbit [Orr & Stickland, 1938], so its specificity to tumours is by no means complete. Recently the criticism has been made [Berenblum *et al.* 1940] that the metabolism of a tumour has in no case been compared with that of the normal tissue from which it is derived, because the normal tissues in question cannot be obtained in sufficient quantity for use with the apparatus so far available. With their new micro-apparatus they have shown that skin epithelium has a metabolism qualitatively the same as that of tumours derived from it, and suggest that the same may be true of all tumours.

Our present results, if confirmed, prove on the contrary that liver cells at any rate do show a sudden change in the type of their metabolism when they form tumours.

SUMMARY

Contrary to the findings of Nakatani *et al.* [1938] we have not observed any change in the glycolytic metabolism of liver tissue in the precancerous phase of butter yellow treatment.

On the other hand, the tumours produced by this agent show a qualitative difference in metabolism from that of normal and cirrhotic liver, the substrate for glycolysis being glycogen in liver tissue and glucose in the tumours derived from it.

The tumours show an aerobic glucolysis equivalent, on the average, to rather more than one-third of the anaerobic glycolysis.

REFERENCES

Ando (1938). *Gann*, **32**, 252.
Berenblum, Chain & Heatley (1940). *Amer. J. Cancer*, **37**, 367.
—— Kendal & Orr (1936). *Biochem. J.* **30**, 709.
Dickens & Greville (1932). *Biochem. J.* **26**, 1546.
—— & Weil-Malherbe (1936). *Biochem. J.* **30**, 659.
Friedemann, Cotonio & Schaffer (1927). *J. biol. Chem.* **73**, 335.
Fujita (1928). *Biochem. Z.* **197**, 175.
György, Keller & Brehme (1928). *Biochem. Z.* **200**, 356.
Hynd & Rotter (1930). *Biochem. J.* **24**, 1390.
Kinosita (1937). *Trans. Soc. Path. jap.* **27**, 665.
Nakatani, Nakano & Ohara (1938). *Gann*, **32**, 240.
Orr (1940). *J. Path. Bact.* **50**, 393.
—— & Stickland (1938). *Biochem. J.* **32**, 567.
Rosenthal (1929). *Biochem. Z.* **207**, 263.
—— (1930). *Biochem. Z.* **227**, 354.
—— & Lasnitzki (1928). *Biochem. Z.* **196**, 340.
Sasaki & Yoshida (1935). *Arch. path. Anat.* **295**, 175.
Warburg (1924). *Biochem. Z.* **152**, 51.
—— Posener & Negelein (1924). *Biochem. Z.* **152**, 309.
Yoshida (1932). *Proc. imp. Acad. Tokyo*, **8**, 464.

50. THE EFFECT OF THIAMINE (VITAMIN B_1) ON FERMENTATION OF YEAST

By H. LASER

From the Molteno Institute, University of Cambridge

(*Received 26 March 1941*)

THIAMINE (vitamin B_1) is known to affect the metabolism and growth of yeast. It was found to stimulate the growth in concentrations as low as 0·01 μg./ml. of culture medium [Williams & Roehm, 1930]. Not every type of yeast, however, is affected in this way, some yeasts responding only very little to thiamine under the usual growth conditions. Similarly some yeasts only are capable of synthesizing thiamine from comparatively simple media [for lit. see Williams, 1941]. Closely related to the effect of thiamine on growth is its effect on yeast fermentation. This is increased by doses of thiamine of the same order of magnitude as those which stimulate growth [Schultz *et al.* 1937, 1, 2]. A quantitative test for determining such minute amounts by the yeast fermentation method has been described by Atkin *et al.* [1939] who found that the addition of 0·01–0·04 μg. of thiamine per 5 mg. yeast (wet weight) suspended in 3 ml. of medium considerably raises the anaerobic CO_2 output during the 2nd hr. of incubation, and that the increase of fermentation caused by doses of 0·01 to 0·02 μg. is proportional to the amount of added thiamine.

In this paper the experiments described by Atkin *et al.* [1939] have been repeated and some more facts have been gained on the effect of thiamine on yeast fermentation.

EXPERIMENTAL

The experiments were made with ordinary Warburg manometers (conical flasks of about 18 ml.) at 20 and 39°. N_2 was freed from traces of O_2 by passing it over heated copper.

For each vessel 1–5 mg. wet weight of ordinary baker's yeast or *Torula utilis* were used, suspended in the medium employed by Atkins *et al.* Their experimental procedure was generally followed except that the volume of fluid per vessel was always kept constant when different amounts of thiamine were tested (*vide* Table 1). The percentage increase in the rate of fermentation caused by thiamine above that of the control to which no thiamine had been added was plotted against concentration of thiamine.

Table 1

Exp. 12, Fig. 4. Period, 60–110 min. Anaerobic fermentation, 3 mg. yeast per vessel, 20°. Thiamine solution, 0·04 μg./ml. Thiamine test solutions: A, 0·02 μg./ml. B, 0·03 μg./ml.

	I	II	III	IV	V
Distilled water	2·0	1·0	1·75	1·0	1·0
Thiamine solution	—	1·0	0·25	1·0 A	1·0 B
Yeast susp.	1·0	1·0	1·0	1·0	1·0
h (mm.)	+41·0	+81·0	+63·75	+74·0	78·75
k_{CO_2}	1·887	1·893	1·903	1·801	1·823
μl. CO_2	+77·5	+153	+121	+133	+143
% increase above I	—	+97·8 %	+56·5 %	+71·9 %	+85·5 %

Results

Anaerobic fermentation of yeast. Fig. 1 shows the percentage increase of anaerobic fermentation at 20° of 5 mg. yeast per vessel for different periods from 0 to 150 min. induced by thiamine in concentrations of 0·01, 0·02 and 0·04 µg. per vessel. The experiment shows:

(1) There is a very marked increase of anaerobic fermentation under the influence of thiamine.

(2) The percentage increase is in definite relation to the amount of thiamine added.

(3) The absolute amount of CO_2 produced under the influence of thiamine increases with time, the relative percentage increase caused by different thiamine concentrations, however, remains more or less the same for each period.

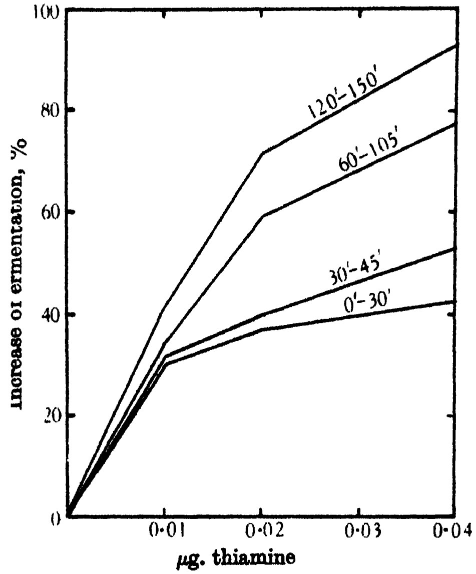


Fig. 1.

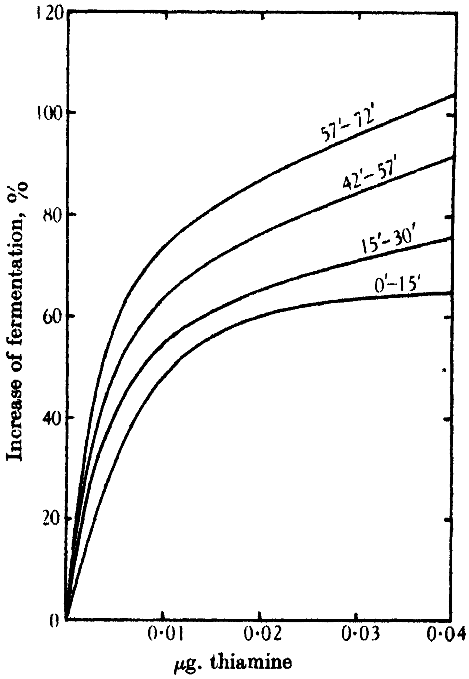


Fig. 2.

Fig. 1. Percentage increase of anaerobic fermentation for different periods of the experiment. 5 mg. yeast (wet wt.) per vessel; 20°.

Fig. 2. Percentage increase of anaerobic fermentation for different periods of the experiment. 2 mg. yeast; 39°.

This last fact probably explains why Atkin *et al.* have used the 2nd hour for their determinations, for it is only in this period that the absolute increase of fermentation caused by a given amount of thiamine is significant enough to reveal comparatively small differences of the fermentation rate caused by different amounts of thiamine.

Fig. 2 shows the percentage increase of anaerobic fermentation by 2 mg. yeast per vessel for different periods from 0 to 72 min. with 0·01, 0·02 and 0·04 µg. thiamine at 39°. On the whole, this experiment closely resembles that of Fig. 1, the same relative increase of fermentation being caused by various amounts of thiamine; and the rate of fermentation increasing with time. The only difference from Fig. 1 is that all the curves lie on a slightly higher level. This represents the temperature coefficient of the effect, which is evidently low, considering that the temperature difference is about 20°. The higher level of fermentation rate in this experiment does not seem to be caused by the fact that the same amount of thiamine is allowed to act on less yeast per vessel (2 mg. instead of 5 mg.) because the same thiamine concentration acting on half the amount of yeast (1 mg., Fig. 3) but at the same temperature (39°) does not further increase the rate of fermentation. Fig. 2 shows also that the quantitative testing of thiamine need not be restricted to the narrow range between 0·01 and 0·02 μg. where the increase of fermentation is proportional to the amount of added thiamine. If the rates of fermentation are plotted against concentration of thiamine for different consecutive periods of the experiment a similar type of curve is obtained, which allows testing for thiamine over a wider range.

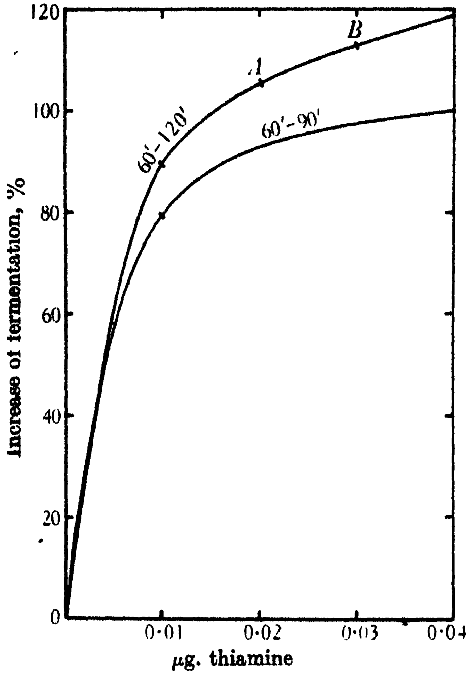


Fig. 3.

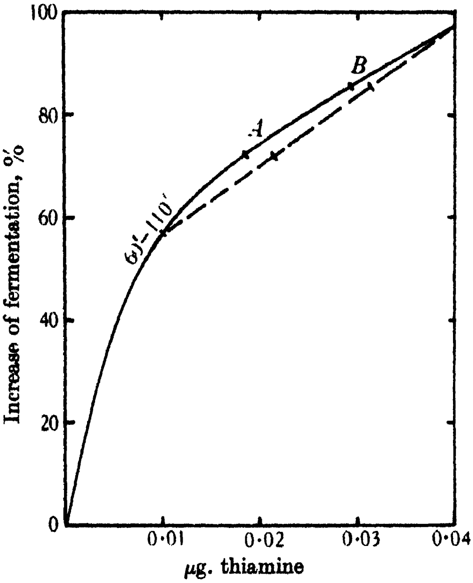


Fig. 4.

Fig. 3. Determination of the thiamine contents of two test solutions, $A = 0{\cdot}02$ μg./1·0 ml., $B = 0{\cdot}03$ μg./1·0 ml., by the percentage increase of anaerobic fermentation. 1 mg. yeast; 39°.

Fig. 4. Same as Fig. 3. 3 mg. yeast; 20°.

Fig. 3 represents the results of such an experiment, in which the strength of two thiamine solutions (*A* and *B*) was determined. Solution *A*, containing 0·02 mg./1·0 ml., and *B*, containing 0·03 μg./1·0 ml., had been given to me without my knowing their thiamine contents. Two consecutive determinations of the fermentation rates were made for the periods 60–90 min. and 60–120 min. with 0·01 and 0·04 μg. thiamine each. The thiamine contents of solutions *A* and *B* were determined by the fermentation rate of the same amounts of yeast for the period 60–120 min. The figure shows that the rates found for *A* and *B* correspond exactly to the amounts of thiamine present, i.e. 0·02 and 0·03 μg.

Fig. 4 (3 mg. yeast; 20°) shows a similar experiment where for the period 60–110 min. the fermentation rate was measured with 0·01 and 0·04 μg. as standard and *A* (0·02 μg.) and *B* (0·03 μg.) as unknown thiamine concentrations. Irrespectively of whether the points marking the fermentation rate with 0·01 and 0·04 μg. were connected by a curve or a straight line, the values obtained for *A* and *B* lie close enough to either of them to allow the respective thiamine contents to be determined sufficiently accurately for all practical purposes.

Aerobic fermentation of yeast. This is quantitatively affected by thiamine in the same way as anaerobic fermentation, but the test can, of course, be made only with yeast varieties which normally have some aerobic fermentation. An example is shown in Fig. 5, where the percentage increase of aerobic fermentation is determined with 0·01, 0·02, 0·03 and 0·04 μg. thiamine, the points marking the fermentation rates with 0·02 and 0·03 μg. lying close to either a curve or a straight line connecting the points which correspond to the fermentation rate with 0·01 and 0·04 μg. of thiamine.

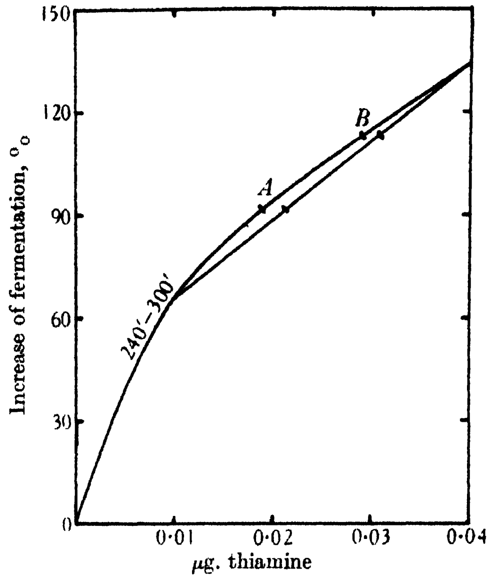


Fig. 5. Determination of the thiamine contents of two test solutions, *A* = 0·02 μg./ml., *B* = 0·03 μg./ml. by the percentage increase of *aerobic* fermentation. 3 mg. yeast; 20°.

Technically the measurement of aerobic fermentation is simpler and quicker than that of anaerobic fermentation. It requires, however, in addition to the usual manometer (I), another manometer (II) containing KOH in the inner cup for the measurement of respiration (x_{O_2}) during the experimental period. Assuming R.Q. = 1, the respiratory $CO_2 = -x_{O_2}$. If the observed pressure changes and vessel constants are denoted by dashes for their respective vessels, and the aerobic fermentation by x_{FCO_2}, then

$$x_{FCO_2} = k'_{CO_2}\left(h' - \frac{h'' k''_{CO_2}}{k'_{O_2}}\right) + h'' k''_{O_2}.$$

Not every type of yeast responds in the manner described when thiamine is added, and yeast which does respond to the addition of thiamine may differ widely with regard to the time at which the increase of fermentation occurs. Some samples of baker's yeast have been tested at which the characteristic curves have been obtained only after 6–7 hr. incubation with thiamine. In such cases the increase of fermentation by thiamine (0·01–0·04 μg.) during the first hours of the experiments was either absent or more or less uniform at between 10 and

20 %, thus not permitting any distinction between the various amounts of thiamine. After 6–7 hr. the different rates became more and more distinct. In some cases incubation was extended for 24 hr., by which time the differences were very marked. The maximum increase of fermentation caused by the addition of 0·04 μg. seldom exceeded 120–140 % however.

Behaviour of Torula utilis. *Torula utilis* did not respond at all within the period examined (24 hr.) to the applied range of thiamine (0·01–0·04 μg.). Neither aerobic nor anaerobic fermentation is increased above that of the control. On the other hand the rate of anaerobic fermentation of the control without thiamine increases steadily with time by approximately 20–25 % per hr. until it reaches a maximum of approximately 600 % at which level it seems to remain steady. *T. utilis*, without the addition of thiamine, behaves with regard

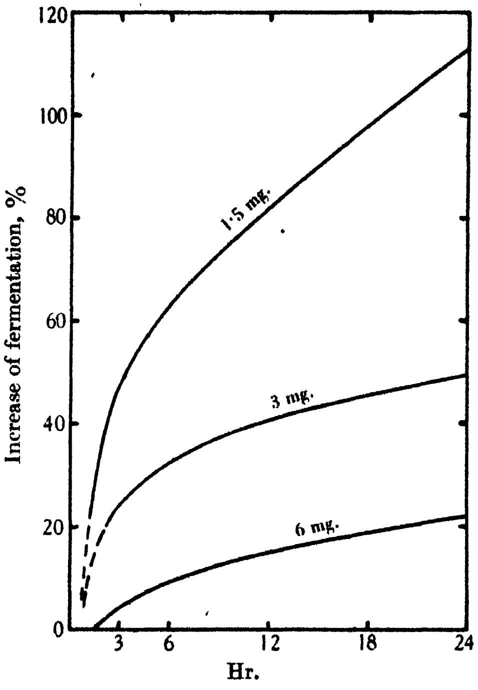


Fig. 6. Percentage increase of anaerobic fermentation caused by the same amount of thiamine (0·04 μg.) acting on different amounts of yeast. 20°.

to its anaerobic fermentation like baker's yeast to which thiamine has been added. This fact seems to justify the conclusion that *T. utilis* is able to synthesize thiamine from the medium and to do this at such a rate that added thiamine (0·01–0·04 μg.) does not further increase the already naturally increasing rate of fermentation. When 0·4 μg. of thiamine was added an increase of fermentation of between 20–30 % was found only after more than 24 hr. incubation. Quantitative tests at this stage of incubation and with these larger doses have, however, not been made.

Torula has only a very small, practically insignificant, aerobic fermentation. Unlike the anaerobic fermentation, this does not increase with time. On the other hand respiration increases at about the same rate as anaerobic fermentation.

While the rate of anaerobic fermentation of *Torula* without thiamine increases, that of baker's yeast as a rule decreases slightly with time. In analogy to the behaviour of *Torula* which probably synthesizes thiamine it might be concluded that baker's yeast is not only incapable of doing this but is losing some of its own preformed thiamine under the conditions of the experiment.

If this is the case, then different amounts of yeast incubated for long periods with the same amount of thiamine should show differences in the increase of CO_2 production. In the experiment summarized in Fig. 6 a sample of baker's yeast was used which responded only very slowly and which reacted to different amounts of thiamine in the characteristic way only after prolonged incubation. Different amounts (1·5, 3 and 6 mg.) of this yeast were exposed to the same amount of thiamine (0·04 μg.) and the relative increase of CO_2 production determined at different times. Since the volume of fluid was the same for each vessel, the relative amounts of added thiamine per mg. of yeast were in the ratio of 1 : 2 : 4. If preformed thiamine was used up at the same rate in each vessel, then the relative increase with time of CO_2 production caused by 0·04 μg. should be greater with 1·5 mg. than with 6 mg. yeast. This is actually the case, as can be seen from Fig. 6.

The effect of reduced thiamine

Lipmann [1936], who studied the reduction of vitamin B_1 by $Na_2S_2O_4$ and by H_2 in presence of platinum black, remarked on the close similarity of his results to those of Warburg *et al.* [1935] with coenzyme. He suggested that the sulphite-reduced compound corresponds to the naturally reduced component of thiamine in the body and assumed that thiamine in the body is reduced and reoxidized. It was therefore of interest to compare the influence of the oxidized and reduced product on the rate of fermentation of yeast. The following results have been obtained.

(1) The reduction of thiamine by H_2+platinum black yields an irreversibly inactive product, e.g. fermentation in presence of H_2-reduced thiamine, even over prolonged periods, is of exactly the same magnitude as that of the control without thiamine.

(2) When thiamine which has previously been reduced by $Na_2S_2O_4$ (in 0·2 % $NaHCO_3$) is added to a readily reacting type of yeast in N_2 (by tipping it from a side bulb) no difference is found as compared with the effect of the oxidized compound. It must be assumed, therefore, that reduced thiamine is rapidly reoxidized within the yeast cells. The smallest amount of yeast used for these experiments was 1·5 mg. with 0·04 μg. reduced thiamine. If still smaller amounts of yeast could be used, a time lag for the complete reactivation of the reduced thiamine might be expected and a quantitative relation established. The technical difficulties, however, in determining this relation seemed unsurmountable: this is not surprising considering that the analogous reoxidation of reduced coenzyme by flavin comes to a standstill within 5 min.

It may be added that $Na_2S_2O_4$ is itself not without influence on the metabolism of yeast. Even in very small amounts it inhibits respiration and fermentation, the latter to a greater extent. No effect on either respiration or fermentation was obtained when 0·01 mg. or less $Na_2S_2O_4$ was added per 3 ml. of fluid. The reduction of thiamine was therefore carried out in a relatively concentrated solution of thiamine, e.g. 1 ml. 0·2 % $NaHCO_3$ contained 10 μg. thiamine and 2·5 mg. $Na_2S_2O_4$. This was diluted 50 times, and 0·2 ml. was then tipped from the side bulb of the vessel into the yeast suspension under anaerobic conditions. The same amount of equally diluted $NaHCO_3$ solution was tipped into the control vessels without thiamine and with oxidized thiamine.

CONCLUSIONS AND SUMMARY

1. Under the conditions used by Schultz *et al.* thiamine in amounts as low as 0·01 μg. increases the anaerobic fermentation of yeast.
2. The percentage increase of the rate of fermentation by 0·01–0·04 μg. thiamine, plotted against thiamine concentration for several consecutive periods of the experiment, gives a similar type of curve. Thus it is possible to make quantitative tests of unknown concentrations of thiamine within this range with considerable accuracy.
3. Not every type of yeast responds with increased fermentation to the addition of thiamine. Thus baker's yeast was found to react regularly and fairly well, while *Torula utilis* under similar conditions did not respond at all.
4. The time at which responding yeast begins to show an increased fermentation after thiamine has been added varies considerably. Some samples react immediately, some only after several hours of incubation.
5. Once the rise of fermentation has started it increases with time for the same dose of thiamine. Differences in the increase of fermentation caused by different amounts of thiamine acting on the same amount of yeast are therefore more pronounced (and easier to evaluate quantitatively) after some incubation, although the relative increases remain the same.
6. In yeasts which react by an increase of anaerobic fermentation, aerobic fermentation is also increased quantitatively by the same minute amounts of thiamine, the percentage increase being of about the same magnitude as that of anaerobic fermentation.
7. The reaction which causes the increase of fermentation has a low temperature coefficient.
8. Different amounts of a slowly reacting sample of yeast incubated with the same amount of thiamine for 24 hr. show, as time goes on, a relatively greater increase of fermentation with decreasing amounts of yeast.
9. Reduction of thiamine by H_2 in presence of platinum black yields an irreversibly inactive product.
10. The $Na_2S_2O_4$-reduced thiamine, when added to yeast in N_2, affects fermentation in the same way as the oxidized form. It is concluded that the reduction by $Na_2S_2O_4$ is reversible and that thiamine reduced by $Na_2S_2O_4$ is reoxidized within the living yeast cell.

This investigation was suggested by Dr A. C. Thaysen to Prof. D. Keilin. I wish to express my thanks to Prof. Keilin for his interest and advice during the course of the experiments.

REFERENCES

Atkin, Schultz & Fry (1939). *J. biol. Chem.* **129**, 471.
Lipmann (1936). *Nature, Lond.*, **138**, 1097.
Schultz, Atkin & Fry (1937, 1). *J. Amer. chem. Soc.* **59**, 948.
——— ——— ——— (1937, 2). *J. Amer. chem. Soc.* **59**, 2457.
Warburg, Christian & Griese (1935). *Biochem. Z.* **282**, 157.
Williams (1941). *Biol. Rev.* **16**, 49.
——— & Roehm (1930). *J. biol. Chem.* **87**, 581.

Note added 26 May 1941. The application of the yeast fermentation method for the quantitative assay of vitamin B_1 in foodstuffs and tissues is limited by the fact that some yeasts are capable of synthesizing vitamin B_1 from certain degradation or split products of the vitamin molecule. These products, however, are not biologically active in animals. The presence of some such products might therefore give a higher fermentation test than warranted by the presence of free thiamine. I am indebted to Dr L. Harris who kindly drew my attention to this point.

51. THE FORMATION OF SUCCINIC ACID IN YEAST

By ARNOŠT KLEINZELLER

From the Department of Biochemistry, University of Sheffield

(*Received 26 March 1941*)

PASTEUR [1860] was the first to observe succinic acid among the substances formed during alcoholic fermentation. In his experiments the yield of succinic acid was 0·5–0·7 % of the fermented sugar. Ehrlich [1909] found that fermenting yeast is capable of converting glutamic acid into succinic acid and advanced the view that all the succinic acid formed during fermentation is derived from glutamic acid; the latter was assumed to arise from the autolysis of dead yeast cells. This view has been widely accepted.

It will be shown in this paper that in addition to Ehrlich's mechanism there is a second mechanism of succinic acid formation in which sugar is the parent substance. Under normal conditions most, if not all, of the succinic acid is derived from this source.

EXPERIMENTAL

Methods. Suspensions of yeast in 1 % KH_2PO_4 or 0·1 *M* phosphate buffer *p*H 7·4, or 0·5 % $NaHCO_3$ were used, and 3 ml. of the suspension containing 2–40 mg. yeast (dry weight) were measured into the main compartment of conical manometer cups. The substrate was measured into the side-arm and the volume brought up to 4 ml. with water unless otherwise stated. The gas space contained air, or expired air (i.e. 5 % CO_2 in air), or, in anaerobic experiments, CO_2. In the anaerobic experiments yellow phosphorus was placed in the centre chamber to remove traces of O_2.

The manometers were shaken at 25°. When equilibrium was established the substrates were added from the side-arm and the cups were shaken until the CO_2 output had almost ended. The yeast suspension was then deproteinized with tungstic acid and the succinate was estimated manometrically [see Krebs, 1937]. The results are expressed in μl. as usual.

In most of the experiments baker's yeast was used, but a few experiments were carried out with brewer's yeast, washed suspensions of pure cultures of *Torulopsis utilis*, or a strain of *Saccharomyces cerevisiae* adapted to the use of galactose (received from Dr Marjory Stephenson, see Stephenson & Yudkin [1936]).

(1) *Effect of media.* Two factors were found to affect the formation of succinate by fermenting yeast: the concentrations of H^+ and of CO_2 and bicarbonate.

Generally no succinate was formed when 1 % KH_2PO_4 was used as a medium, whilst 85–245 μl. succinate were formed from 50 mg. of glucose when 0·5 % $NaHCO_3$ saturated with 5 % CO_2 was used. The importance of the CO_2 pressure is demonstrated by Exps. 1 and 2, Table 1, where two media of approximately equal *p*H but of different CO_2 concentration were compared. No succinate was formed in the absence of glucose.

Table 1. *Succinate formation by baker's yeast in different media.*

(1 ml. 5 % glucose; 3 ml. medium.)

No.	mg. yeast (dry wt.)	Medium	µl. succinate found
1 and 2	18·6	1 % KH_2PO_4; air; CO_2 absorbed by NaOH	0; 0
		0·1 *M* phosphate buffer *p*H 7·4; air; CO_2 absorbed by NaOH	0; 0
		0·5 % $NaHCO_3$; 5 % CO_2 in air	85; 92
3	19·5	1 % KH_2PO_4; air; CO_2 absorbed by NaOH	37
		0·5 % $NaHCO_3$; 5 % CO_2 in air	152
		0·5 % $NaHCO_3$; deproteinized immediately	15
4	10·0	1 % KH_2PO_4; CO_2	0
	20·0	1 % KH_2PO_4; CO_2	0
	40·0	1 % KH_2PO_4; CO_2	0
	10·0	0·5 % $NaHCO_3$; CO_2	108
	20·0	0·5 % $NaHCO_3$; CO_2	109
	40·0	0·5 % $NaHCO_3$; CO_2	119

(2) *Effect of glucose.* The amount of succinate formed during the fermentation in the presence of $NaHCO_3$ and CO_2 is proportional to the quantity of sugar fermented; it increases linearly with the amount of sugar added and with time. Per 100 mol. of sugar fermented about 1·35–3·96 mol. of succinate are formed. Slightly larger quantities seem to be formed when low concentrations of yeast are used (see Table 2).

The proportion of yeast present to succinate formed, which would be expected to be fairly constant if Ehrlich's hypothesis were correct, shows very wide variations (Table 1, Exp. 4). If a small quantity of yeast is used, the yield of succinate exceeds the total dry weight of yeast (Table 2, Exp. 3). This is conclusive proof of the formation of succinate from added glucose.

Table 2. *Succinate formation from glucose*

Baker's yeast in 3·0 ml. 0·5 % $NaHCO_3$. 5 % CO_2 in air in Exps. 1 and 2; CO_2 in Exp. 3.

No.	mg. yeast (dry wt.)	Glucose added	Duration of exp. (hr.)	µl. succinate formed
1	23·4	0	23	0
		1·0 ml. 5 %	6	134
		1·0 ml. 10 %	8	274
		2·0 ml. 10 %	23	434
2	19·5	0	0	15
		1·0 ml. 5 %	1	19
		1·0 ml. 5 %	3	100
		1·0 ml. 5 %	5	152
3	2·0	1·0 ml. 10 %	90	407 (=2·14 mg.)

(3) *Effect of* O_2. The yield of succinate was found to be the same in aerobic and anaerobic experiments, although fermentation is more rapid in the absence of O_2 (Table 3).

Table 3. *Formation of succinate under aerobic and anaerobic conditions*

20·4 mg. baker's yeast (dry weight) in 3 ml. 0·5 % $NaHCO_3$; 1 ml. 5 % glucose.

No.	Gas mixture	µl. succinate found
1	5 % CO_2 in air	137
	CO_2; phosphorus	148
2	5 % CO_2 in air	175
	CO_2; phosphorus	174

(4) *Succinate formation from various sugars.* The amounts of succinate formed from glucose fructose and sucrose were found to be of the same order. This confirms Pasteur's [1860] results. A strain of *Saccharomyces cerevisiae* adapted to the use of galactose formed the same quantities of succinate from glucose and from galactose.

Table 4. *Formation of succinate from various sugars*

Exp. 1. 23·4 mg. baker's yeast in 3 ml. 0·5 % $NaHCO_3$; 5 % CO_2 in air; 1 ml. 5 % sugar added.
Exp. 2. 19·3 mg. *S. cerevisiae* adapted to use of galactose, in 3 ml. 0·5 % $NaHCO_3$; 1 ml. 10 % sugar added; 5 % CO_2 in air.

Exp.	Sugar added	μl. succinate found
1	Glucose	164
	Fructose	190
	Saccharose	184
2	0	0
	Glucose	183
	Galactose	151

Table 5. *Effect of various substrates on the formation of succinate in baker's yeast*

(3 ml. yeast suspension, 1 ml. substrate. Medium, 0·5 % $NaHCO_3$; 5 % CO_2 in air, unless otherwise stated. Brackets indicate experiments carried out on the same suspension and therefore directly comparable.)

mg. yeast	Additions	μl. succinate found
23·4	1·0 ml. 0·2 *M* Na acetate	0
	1·0 ml. 0·2 *M* Ba acetate	0
	1·0 ml. 0·2 *M* Na acetate*	0
	1·0 ml. 0·2 *M* Ba acetate*	0
19·5	0	20
	50 mg. glucose	161·5
	1·0 ml. 0·25 *M* glycerol	6·5
	1·0 ml. 0·25 *M* α-glycerophosphate	19
	50 mg. glucose; 0·5 ml. 0·2 *M* pyruvate	157
23·4	50 mg. glucose	149
	50 mg. glucose; 0·5 ml. 0·2 *M* fumarate	138
	50 mg. glucose; 0·5 ml. 0·2 *M* aspartate	184
	1·0 ml. 0·25 *M* fumarate	29
	1·0 ml. 0·25 *M* aspartate	10
19·5	50 mg. glucose	157
	50 mg. glucose; 0·5 ml. 0·2 *M* oxaloacetate	187
	1·0 ml. 0·25 *M* oxaloacetate	24·5
15·5	50 mg. glucose	132
	50 mg. glucose; 0·5 ml. 0·2 *M* *l*-tartrate	138
	1·0 ml. 0·25 *M* *l*-tartrate	0
19·5	50 mg. glucose	134
	50 mg. glucose; 0·5 ml. 0·2 *M* *l*-malate	194
	50 mg. glucose; 0·5 ml. 0·2 *M* α-hydroxyglutarate	183
	1 ml. 0·25 *M* *l*-malate	45
	1 ml. 0·25 *M* α-hydroxyglutarate	21
19·5	50 mg. glucose	160
	1·0 ml. 0·25 *M* α-ketoglutarate	156
19·5	50 mg. glucose	134
	50 mg. glucose; 0·5 ml. 0·2 *M* glutamate	450
	50 mg. glucose; 0·5 ml. 0·2 *M* α-ketoglutarate	174
19·5	50 mg. glucose	141
	50 mg. glucose; 0·5 ml. 0·2 *M* glutamate	493
	50 mg. glucose; 0·5 ml. 0·2 *M* α-hydroxyglutarate	195
15·5	50 mg. glucose	132
	50 mg. glucose; 0·5 ml. 0·2 *M* arginine	131
	50 mg. glucose; 0·5 ml. 0·2 *M* ornithine	138

* 0·1 *M* phosphate buffer; air.

(5) *Effect of other substrates.* The effect of the following substances on the formation of succinate was tested in the presence and in the absence of glucose: acetate, pyruvate, lactate, glycerol, α-glycerophosphate, oxaloacetate, *l*-malate, fumarate, *l*-tartrate, γ-aminobutyrate, *l*-glutamate, α-hydroxyglutarate, α-ketoglutarate, *dl*-ornithine, *l*-arginine. The final concentration of these substances when added alone was 0·0625*M*, when added with glucose 0·025*M* (unless otherwise stated).

In absence of glucose α-ketoglutarate was the only substance to yield succinate [see Neuberg & Ringer, 1915]. In presence of glucose 2- and 3-carbon compounds had no effect on the formation of succinate. Oxaloacetate, *l*-malate and aspartate increased the yield of succinate by about 20 % and α-ketoglutarate and α-hydroxyglutarate about 30 %, whilst fumarate, *l*-tartrate, γ-aminobutyrate, arginine and ornithine had no effect (Table 5).

The succinate formation from *l*(+)-glutamic acid, first shown by Ehrlich [1909], is confirmed. The quantity of succinate formed depends on the composition of the medium; only a small amount is formed when the medium is weakly acid and CO_2 is absorbed by NaOH; considerable quantities appear in 0·5 % $NaHCO_3$ saturated with 5 % CO_2.

No difference was found in the yield of succinate from glutamic acid under aerobic or anaerobic conditions.

Table 6. *Formation of succinate from* l(+)*-glutamate in baker's yeast*

Glutamate concentration *M*/40 unless otherwise stated.

No.	mg. yeast	Substrates		Medium	μl. succinate found
		Glucose (50 mg.)	Glutamate		
1	23·4	+	−	0·5 % $NaHCO_3$; 5 % CO_2 in air	157
		+	+	,, ,,	500
		−	+*	,, ,,	6
2	18·6	+	−	0·5 % $NaHCO_3$; 5 % CO_2 in air	82
		+	+	,, ,,	402
		+	+	0·1 *M* phosphate buffer; air; CO_2 absorbed by NaOH	0
		+	+	1 % KH_2PO_4; air; CO_2 absorbed by NaOH	0
3	18·6	+	−	0·5 % $NaHCO_3$; 5 % CO_2 in air	92
		+	+	,, ,,	396
		+	+	0·1 *M* phosphate buffer; air; CO_2 absorbed by NaOH	0
		+	+	1 % KH_2PO_4; air; CO_2 absorbed by NaOH	0
4	20·4	+	−	0·5 % $NaHCO_3$; 5 % CO_2 in air	137
		+	+	,, ,,	305
		+	+	0·5 % $NaHCO_3$; CO_2; phosphorus	330

* 0·0625 *M* glutamate.

Table 7. *Effect of* NH_4Cl *on the formation of succinate in baker's yeast*

16·1 mg. yeast in 3 ml. 0·5 % $NaHCO_3$; 5 % CO_2 in air; 50 mg. glucose.

Additions (final conc. 0·025*M*)	μl. succinate found
0	82
l-Glutamate	364
α-Ketoglutarate	250
α-Hydroxyglutarate	141·5
α-Ketoglutarate; NH_4Cl	78
α-Hydroxyglutarate; NH_4Cl	83
NH_4Cl	92

α-Ketoglutarate and α-hydroxyglutarate have been assumed to be intermediates in the succinate formation from glutamate [Ehrlich, 1909; Neuberg & Ringer, 1915] and it is therefore remarkable that under identical conditions the yields of succinate from these acids were smaller than those from glutamate. This result is not affected by the addition of equimolecular quantities of NH_4Cl (Table 7). Since the permeability of the yeast cell may influence the rate of reaction we do not regard our results as contradictory to the hypothesis of Neuberg & Ringer.

(6) *Effect of growth.* Little or no succinate was found, even in presence of $NaHCO_3$, when the fermentation proceeded in a medium in which growth occurs. Two media were tested, a modified Czapek-Dox medium (2 g. $NaNO_3$, 1·32 g. $Na_2HPO_4, 2H_2O$, 0·5 g. $MgSO_4, 7H_2O$, 0·5 g. KCl, 0·01 g. $FeSO_4, 7H_2O$ in 1 l. tap water) and the medium of Fink & Krebs [1938] (2·6 g. $(NH_4)_2HPO_4$, 3·3 g. $(NH_4)_2SO_4$, 2 g. K_2SO_4, 1·5 g. $MgSO_4, 7H_2O$, in 1 l. tap water).

The recovery of succinate added to yeast fermenting in 0·5 % $NaHCO_3$, or 1 % KH_2PO_4, was quantitative, whilst a considerable amount of succinate was taken up by the yeast fermenting in the complex medium (Table 8).

Table 8. *Effect of growth on the formation of succinate in baker's yeast*

20·0 mg. yeast in 3 ml. medium; 5 % CO_2 in air.

No.	Medium	Additions: Glucose mg.	Additions: Succinate μl.	Succinate found, μl.
1	Czapek-Dox	—	—	0
	„	50	—	39
	0·5 % $NaHCO_3$	50	—	138
2	Fink-Krebs	—	—	0
	„	50	—	0
	0·5 % $NaHCO_3$	50	—	115
3	0·5 % $NaHCO_3$	50	—	247
	„	50	672	943
	Fink-Krebs	50	—	45
	„	50	672	638

(7) *Experiments on brewer's yeast and on* Torulopsis utilis. Experiments with varying quantities of sugar were carried out on fresh brewer's yeast and on a pure culture of *Torulopsis utilis*. As in the case of baker's yeast the yield of succinate was found to be proportional to the quantity of sugar fermented (Table 9).

Table 9. *Succinate formation from glucose in different yeasts*

Exp. 1. 15 mg. brewer's yeast in 3 ml. 0·5 % $NaHCO_3$; 5 % CO_2 in air; glucose.
Exp. 2. 20·2 mg. *Torulopsis utilis* in 3 ml. 0·5 % $NaHCO_3$; 5 % CO_2 in air; glucose.

No.	mg. glucose added	Duration of exp. (hr.)	μl. succinate found
1	0	0	0
	0	30	10
	50	6½	87
	100	24	167
	200	30	268
2	0	22	0
	25	2½	58
	50	4	107
	100	6½	195
	200	22	236

(8) *Presence of fumarase in yeast.* In view of the fact that fumarase takes part in the synthesis of succinate in some organisms [Krebs & Eggleston, 1940; Smyth, 1940] it was thought of interest to examine the fumarase activity of yeast. Fumarate was estimated according to Krebs *et al.* [1940]. No evidence was found of the presence of fumarase in baker's yeast. Added malate was not converted into fumarate within *p*H 5–8. This explains why malate and fumarate behave differently (Table 5). The conversion of malate into succinate must be regarded as a direct reduction which does not involve the formation of fumarate.

DISCUSSION

(1) *Source of succinate.* The experimental results show that in normal conditions of fermentation sugar is the main parent substance of succinate (Exp. 5, Table 1). The fact that 1 mg. of yeast can form 1·07 mg. succinic acid proves that the succinate cannot be derived from the yeast protoplasm, as assumed by Ehrlich. It must be formed from the added substrate, i.e. glucose.

(2) *Mechanism of succinate formation from glucose.* The formation of succinate depends on the presence of $NaHCO_3$ and of CO_2. A similar relationship between the formation of C_4-dicarboxylic acids and the presence of $NaHCO_3$ was observed by Elsden [1938] and by Krebs & Eggleston [1940], who concluded that oxaloacetic acid is synthesized in animal tissues by condensation of pyruvic acid with CO_2. It is therefore suggestive to assume that succinic acid in yeast is formed in a similar way. The 'carboxylation' of pyruvic acid would represent a side reaction in the metabolism of pyruvate, the main reaction being the decarboxylation of pyruvic acid. In accordance with this view are the observations that oxaloacetate and malate increase the yield of succinate from glucose, and that yeast is able to bind CO_2 [Ruben & Kamen, 1940].

(3) *Formation of succinate from acetate.* According to Table 5 no succinate was formed from acetate (Na or Ba salt), contrary to the results of Wieland & Sonderhoff [1932], Sonderhoff & Deffner [1936], Sonderhoff & Thomas [1937]. It should be noted that our experimental conditions differ in some ways from those of Wieland and his collaborators. In our experiments fresh yeast, as opposed to 'impoverished' yeast, was used and the relatively short experimental times (24 hr.) excluded bacterial infection, which has previously been accepted by Wieland as an explanation of some of his results [Wieland *et al.* 1936].

SUMMARY

1. The amount of succinate formed in resting suspensions of baker's yeast, brewer's yeast and *Torulopsis utilis* is proportional to the amount of sugar fermented. The yield of succinate varies between 1·35 and 3·96 g. mol. per 100 g. mol. of sugar. The quantity of succinate can exceed the dry weight of the fermenting yeast. This shows conclusively that most of the succinate formed during fermentation is derived from the fermented sugar. Ehrlich's view, according to which succinic acid is derived from the glutamic acid of autolysed yeast cells, is therefore to be discarded.

2. The formation of succinate depends on the presence of bicarbonate in the medium. No succinate is formed in 1 % KH_2PO_4; between 0·45 and 1·29 mg. succinate are formed when 50 mg. glucose are fermented by yeast suspended in 0·5 % $NaHCO_3$.

3. The amount of succinate formed from various fermentable sugars is of the same order.

4. Oxaloacetate, *l*-malate and *l*-aspartate increase the yield of succinate in the presence of glucose by about 20 %, α-ketoglutarate and α-hydroxyglutarate about 30 %, *l*-glutamate about 200 %, whilst various other substances had no effect.
5. No succinate is found when glucose is fermented by growing yeast cells.
6. Fumarase is absent from baker's yeast.
7. The mechanism of succinate formation from glucose is discussed.

The author wishes to express his gratitude to Dr H. A. Krebs for having suggested this investigation and for his constant help and advice, and also to the Sheffield Co-ordinating Committee for Refugees and to the International Student Service for a maintenance grant.

REFERENCES

Ehrlich (1909). *Biochem. Z.* **19**, 391.
Elsden (1938). *Biochem. J.* **32**, 187.
Fink & Krebs (1938). *Biochem. Z.* **299**, 1.
Krebs (1937). *Biochem. J.* **31**, 2095.
—— & Eggleston (1940). *Biochem. J.* **34**, 1383.
—— Smyth & Evans (1940). *Biochem. J.* **34**, 1041.
Neuberg & Ringer (1915). *Biochem. Z.* **71**, 226.
Pasteur (1860). *Ann. chim. (Phys.)*, 3^{e} série, **58**, 323.
Ruben & Kamen (1940). *Proc. Nat. Acad. Sci., Wash.*, **26**, 418.
Smyth (1940). *Biochem. J.* **34**, 1598.
Sonderhoff & Deffner (1936). *Liebigs Ann.* **525**, 132.
—— & Thomas (1937). *Liebigs Ann.* **530**, 195.
Stephenson & Yudkin (1936). *Biochem. J.* **30**, 506.
Wieland, Crawford & Walch (1936). *Liebigs Ann.* **525**, 119.
—— & Sonderhoff (1932). *Liebigs Ann.* **499**, 213.

52. NICOTINAMIDE, COZYMASE AND TISSUE METABOLISM

By P. J. G. MANN and J. H. QUASTEL

From the Biochemical Laboratory, Cardiff City Mental Hospital

(*Received 1 April 1941*)

During the course of experiments on the respiratory activities of intact, minced and ground mammalian tissues we have found that there exists in brain and in liver a very active mechanism which accomplishes the breakdown of cozymase under both aerobic and anaerobic conditions. We have also found that this mechanism can be inhibited in a specific manner by nicotinamide. A preliminary note incorporating these results has already been published [Mann & Quastel, 1941]. The following communication is concerned with a description in detail of these and further results, together with observations on the effects of nicotinamide on the inhibitory activity of cobra venom in respiratory systems.

Technique

Measurements of respiratory activity

The Warburg apparatus has been used for the measurement of rates of O_2 uptake, or of CO_2 output when using the anaerobic technique of Quastel & Wheatley [1938]. The volume of fluid in the respiratory vessel was always maintained at 3 ml. and experiments were at 37° for periods usually not longer than 1 hr.

Preparation of biological material

Ground tissues. In our first experiments with ground brain, we used rat brain tissue (1 part brain to 10 parts 0·9 % NaCl) which had been finely ground in the wet-crushing mill of Booth & Green. This gave an excellent preparation having a high activity in breaking down cozymase. Subsequently, however, it was found unnecessary to use the wet-crushing mill, since a very active preparation could be made simply by grinding the tissue with sand in the presence of 0·9 % NaCl. The finely ground tissue was squeezed through muslin and the extract diluted with 0·9 % NaCl so that 10 ml. of the fluid contained 1 g. of the original tissue. This technique has been adopted for the preparation of ground tissues of rat brain, rat liver and rat kidney.

Muscle extract. Dialysed skeletal (rabbit) muscle extract as a source of cozymase-free lactic dehydrogenase was prepared by the method of Green *et al.* [1937].

Succinic and lactic dehydrogenases. The preparation made from washed pig's heart by the method of Keilin & Hartree [1938] sufficed as a source of both these enzymes. In some experiments extract of skeletal muscle or suspension of rat kidney was used as a lactic dehydrogenase.

Heart flavoprotein. This was prepared by a method similar to that described by Straub [1939].

Cozymase. A partially purified preparation, originally obtained from yeast, was used without further purification, as a high degree of purity was not required. Its activity was frequently tested by appropriate control experiments.

Media

All media in the aerobic experiments contained 0·02*M* Na phosphate buffer *p*H 7·4 and all reagents were neutralized so that the *p*H of the fluid in the manometric vessel was 7·4. When tissue slices were investigated a phosphate-Locke medium was used. This contained 0·02*M* Na phosphate *p*H 7·4, 0·004*M* K^+ and 0·002*M* Ca^{++}. An atmosphere of air was used in all aerobic experiments in which tissue slices were not involved; when intact slices were used air in the manometric vessels was displaced by O_2. In anaerobic experiments, using the ferricyanide technique, phosphate was omitted. The medium consisted of 0·025*M* $NaHCO_3$ together with the other constituents under investigation. The atmosphere was 95 % N_2 + 5 % CO_2. 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was placed in the side tube and tipped into the main vessel after temperature equilibration. For further details of this technique see Quastel & Wheatley [1938].

In most experiments either 0·1 or 0·2 ml. of the cozymase solution was placed in the side tube of the manometric vessel, and after temperature equilibration had been attained this was tipped into the main vessel containing the tissue and respiratory constituents. Any variation in this procedure will be made clear in the text.

RESULTS

The effects of addition of ground brain suspensions on the lactic dehydrogenase activities of muscle and kidney

The addition of small quantities of ground rat brain suspension to a lactic dehydrogenase system prepared from mammalian tissues results in a profound fall in the activity of the system. This is shown using systems prepared from rabbit skeletal muscle, ground pig heart muscle and ground rat kidney. With rabbit skeletal muscle a system is prepared similar to that used by Corran *et al.* [1939]. This consists of dialysed rabbit skeletal muscle extract (which is cell-free), pig heart flavoprotein, Na lactate, NaCN, methylene blue and cozymase. This system as shown by Corran *et al.* [1939] takes up O_2, pyruvate being formed and fixed by the cyanide. Hydrogen from the lactate passes eventually to O_2 through the chain of cozymase, flavoprotein and methylene blue. The addition to the system of 0·2 ml. of a rat brain suspension may reduce by 50 % the rate at which it consumes O_2. Typical results are shown in Table 1 and in Fig. 1. When a suspension of washed ground pig heart muscle is used as a source of lactic dehydrogenase there is no necessity to add flavoprotein as the suspension appears already to possess ample quantities. The feeble O_2 uptake secured by a mixture of washed ground pig heart muscle, Na lactate, NaCN and methylene blue is much increased by the addition of cozymase. The addition however of a mixture of cozymase and 0·2 ml. rat brain suspension reduces this rate of O_2 uptake to a value less than that which obtains in the absence of added cozymase (see Table 1). A ground rat kidney suspension provides a good source of a lactic dehydrogenase preparation relatively free from cozymase. Such a suspension, when used in small quantities, takes up but little O_2 in the presence of Na lactate, NaCN and methylene blue. The addition of cozymase powerfully stimulates the rate of O_2 uptake (Table 1, (3) and (4)). The further addition of a small quantity of ground rat brain suspension reduces the oxidative activity to very small values. This is illustrated by the results shown in Table 1.

Table 1. *Effects of ground brain suspension on lactic dehydrogenase activity*

Brain suspension was prepared by grinding finely 3·1 g. fresh rat brain in 31 ml. 0·9 % NaCl. Lactic dehydrogenase system consisted of lactic dehydrogenase preparation, pig heart flavoprotein (0·5 ml.), sodium lactate (0·13 *M*), sodium cyanide (0·08 *M*), phosphate buffer (0·02 *M*) *p*H 7·4, methylene blue and cozymase. Air; 37°.

Source of lactic dehydrogenase	Methylene blue %	Cozymase mg.	Rat brain suspension ml.	µl. O_2 uptake
				In 1 hr.
(1) 0·3 ml. rabbit skeletal muscle extract	0·017	1	0	205·2
		1	0·2	108·9
		1	0·4	64·0
(2) 0·3 ml. washed ground pig heart muscle	0·04	0	0	45·9
		2	0	136·2
		2	0·2*	28·5
(3) 0·2 ml. ground rat kidney suspension	0·04	0	0	33·5
		2	0	179·5
				In 30 min.
(4) 0·2 ml. ground rat kidney suspension	0·04	0	0	30·1
		1	0	66·0
		1	0·2	10·5

* In this experiment the cozymase and rat brain suspension were incubated together for 15 min. at 37° before adding to the main system.

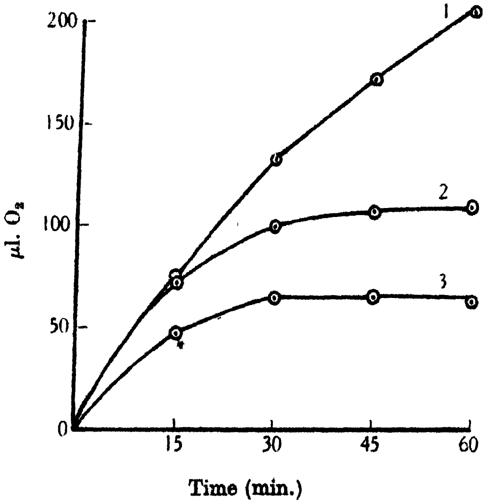


Fig. 1. Effects of ground brain suspensions on oxygen uptake by lactic dehydrogenase system (muscle extract, flavoprotein, lactate, cyanide, cozymase and methylene blue).

1. Lactic dehydrogenase system.
2. Lactic dehydrogenase system + 0·2 ml. ground rat brain suspension.
3. Lactic dehydrogenase system + 0·4 ml. ground rat brain suspension.

Destruction of cozymase by suspensions of ground mammalian tissues

The powerful inhibitory effects of ground brain suspensions on lactic dehydrogenase systems are due to their destruction of cozymase. This is proved by the following facts:

(1) When cozymase is added to a system containing lactic dehydrogenase, a ground brain suspension, lactate, cyanide and methylene blue, the inhibition of

the rate of O_2 uptake due to the brain develops slowly, the rate of development of the inhibition depending on the amount of brain used. Thus, as seen in Table 1 (1) and in Fig. 1, the presence of 0·4 ml. rat brain suspension brings about a much larger inhibition of the rate of O_2 uptake than occurs in the presence of 0·2 ml. brain suspension. If, however, the cozymase is incubated together with 0·2 ml. brain suspension for 15 min. at 37°, before addition to the mixture of lactic dehydrogenase, lactate, cyanide and methylene blue, a large inhibition of the rate of O_2 uptake takes place at once—the mixture behaving in fact as though no cozymase had been added. This is shown by the results given in Table 2. It is clear from these results that both rat brain and rat liver are able to destroy cozymase. However, after the addition of an incubated mixture of rat kidney suspension and cozymase to the lactic dehydrogenase of pig heart, an acceleration instead of an inhibition of O_2 uptake takes place. This is due to the fact that rat kidney has but little ability to destroy cozymase and also provides an extra source of lactic dehydrogenase.

Table 2. *Destruction of cozymase by tissues*

Lactic dehydrogenase system and brain suspension as in Table 1. Liver and kidney suspensions made by grinding tissue with sand and saline (1 g. tissue to 10 ml. saline) and squeezing through muslin. Air; 37°.

Source of lactic dehydrogenase	Methylene blue %	Treatment of cozymase	μl. O_2 uptake in 1 hr.
(1) 0·3 ml. rabbit skeletal muscle extract	0·017	1 mg. added. Untreated	262·8
		1 mg. incubated with 0·2 ml. brain suspension for 15 min. at 37° before addition to lactic dehydrogenase system	44·3
		1 mg. incubated in absence of brain suspension for 15 min. at 37° before addition to lactic dehydrogenase system	272·0
(2) 0·3 ml. washed ground pig heart muscle	0·04	1 mg. added. Untreated	136·2
		1 mg. incubated with 0·2 ml. rat kidney suspension for 15 min. at 37° before addition to lactic dehydrogenase system	243·6
(3) 0·3 ml. rat kidney suspension	0·04	No cozymase added	59·9
		2 mg. added. Untreated	189·4
		2 mg. incubated with 0·2 ml. liver suspension for 15 min. at 37° before addition to lactic dehydrogenase system	57·8

(2) The inhibition of the lactic dehydrogenase-cozymase system by a brain suspension takes place anaerobically, using ferricyanide as oxidant of reduced cozymase instead of flavoprotein and methylene blue (Table 3). This result shows that the inhibition cannot be attributed to a destruction of flavoprotein by the brain suspension.

(3) The inhibitory effect of cozymase on succinic dehydrogenase activity [Keilin & Hartree, 1940] is entirely neutralized by the addition to the system of a small quantity of brain suspension. This result is shown in Table 12 and will be referred to in more detail later.

Anaerobic destruction of cozymase by brain

Cozymase is broken down by a brain suspension under strictly anaerobic conditions. The experiment demonstrating this is carried out by using a Warburg manometric vessel equipped with two side arms. In one side arm is placed the brain suspension, in the other ferricyanide solution. The main vessel contains a lactic dehydrogenase preparation, cozymase, Na lactate, NaCN and $NaHCO_3$ (0·025*M*). The air in the apparatus is displaced by 95 % N_2 + 5 % CO_2, and only after strictly anaerobic conditions are obtained is the brain suspension tipped into the main vessel containing the cozymase. Finally, after temperature equilibration, the ferricyanide is tipped into the main vessel. A control experiment, with no brain suspension present, is also carried out. As the results given in Table 3 show, the effect of the brain suspension under strictly anaerobic conditions is to reduce the activity of the lactic dehydrogenase system.

Table 3. *Anaerobic destruction of cozymase by brain*

Rat kidney suspension was used as source of lactic dehydrogenase. Brain suspension was made up as in Table 1.

Medium. Bicarbonate (0·025*M*)-saline, Na lactate 0·13*M*; NaCN 0·08*M*; 1 mg. cozymase. 95 % N_2 + 5 % CO_2; 37°. Vessels with two side arms were used:

In 1st vessel: 0·2 ml. brain suspension placed in one side arm.
0·2 ml. 8·3 % $Na_3Fe(CN)_6$ placed in other side arm.
Lactic dehydrogenase system in main vessel.

In 2nd vessel: As above, but no brain suspension present.

After anaerobic conditions were secured, the brain suspension was added to the lactic dehydrogenase system in first vessel. After equilibration, the ferricyanide was added to both vessels.

	μl. CO_2 output in 1 hr.*
Lactic dehydrogenase system; no brain suspension added	378·8
Lactic dehydrogenase system; 0·2 ml. brain suspension added	204·9

* Retention of CO_2 due to addition of small volume of brain suspension is here neglected.

This result shows that the destruction of cozymase by the brain suspension is not due to an oxidative breakdown and we conclude, for the time being, that the destruction of the coenzyme is accomplished by a hydrolytic enzyme. We will refer to this as cozymase nucleotidase.

Table 4. *Thermolability of cozymase nucleotidase*

Lactic dehydrogenase system: Washed ground pig heart suspension (0·5 ml.), 0·13*M* Na lactate; 0·02*M* phosphate buffer *p*H 7·4; 0·08*M* NaCN; 0·04 % methylene blue. 1 mg. cozymase. Ground brain suspension used as cozymase nucleotidase. Air; 37°.

	μl. O_2 uptake in 1 hr.
Lactic dehydrogenase system, no cozymase added	34·1
Lactic dehydrogenase system, with cozymase	183·0
Lactic dehydrogenase system + cozymase previously incubated with 0·2 ml. brain suspension for 15 min. at 37°	38·9
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. brain, which had been heated for 5 min. at 70°	161·3
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. brain which had been heated for 5 min. at 100°	173·4

Thermolability of cozymase nucleotidase

Results given in Table 4 show that the enzyme in brain responsible for the breakdown of cozymase is thermolabile. It is completely destroyed by exposure to 100° for 5 min. and almost completely destroyed by exposure to 70° for the same period.

Can the inactivation of cozymase be due to preferential adsorption on brain proteins?

The possibility arises [cf. Lipton & Elvehjem, 1940] that the inactivation of cozymase by a brain extract may not be due to hydrolysis of the coenzyme but to its preferential adsorption on, or combination with, brain proteins. According to this suggestion the coenzyme is not destroyed, but removed from its sphere of action by adsorption on proteins having a high affinity for it.

This problem has been attacked in the following way. Cozymase is incubated together with a brain suspension at 37° for 15 min., when the inactivation of cozymase is complete. The mixture is divided into lots *A*, *B* and *C*. Mixture *A* is left undisturbed. Mixture *B* is heated for 5 min. at 70°. This treatment is known to destroy the inactivating mechanism and presumably, therefore, the proteins responsible for adsorption of the cozymase. Since the cozymase, by hypothesis, is not broken down by adsorption on brain proteins, it should be liberated from the heat-treated proteins. Hence mixture *B*, in contrast to mixture *A*, should contain free cozymase. Mixture *C* is heated for 5 min. at 70° and to this, after cooling, is added free cozymase. If the brain proteins, alleged to be responsible for the adsorption of cozymase, are destroyed by the heat treatment, they will now be unable to inactivate the added cozymase. Hence it should follow if the adsorption theory be correct, that mixture *A* will contain no free cozymase, and that mixtures *B* and *C* will contain free cozymase, *C* being richer in this respect than *B*. On the other hand if the adsorption theory be incorrect, both mixtures *A* and *B* will contain no free cozymase whilst mixture *C* will contain the free cozymase which was subsequently added to it.

Experiment (see Table 5) shows that no free cozymase is liberated from the incubated cozymase-brain mixture after heat treatment and that the heated mixture is unable to inactivate any added cozymase. These facts are in accordance with the view that cozymase is inactivated by breakdown by an enzyme in the brain and not by adsorption on special proteins.

Table 5. *Effect of heating cozymase-brain extract mixture after incubation*

The following mixture was prepared: Ground rat brain suspension (0·6 ml.), cozymase (3 mg.) in 2·4 ml. phosphate buffer (0·02*M*) *p*H 7·4. This was incubated at 37° for 15 min. and divided into three equal lots (*A*, *B*, *C*).

A was not treated further.

B was heated for 5 min. at 70° and cooled.

C was heated for 5 min. at 70°, cooled and to it was added 1 mg. cozymase.

A, *B* and *C* were transferred to Warburg vessels containing a lactic dehydrogenase system (washed ground pig's heart suspension 0·5 ml., Na lactate 0·13*M*, NaCN 0·08*M*, methylene blue 0·04 % and 0·02*M* phosphate buffer *p*H 7·4). Air; 37°.

	μl. O_2 uptake in 1 hr.
Lactic dehydrogenase system containing *A*	36·2
Lactic dehydrogenase system containing *B*	32·2
Lactic dehydrogenase system containing *C*	193·2

Effects of addition of nicotinamide

The addition of nicotinamide to a ground brain suspension has the effect of greatly inhibiting the cozymase nucleotidase. This is shown most clearly by incubating a mixture of brain suspension, cozymase and nicotinamide for

15 min. at 37° and adding the mixture, after incubation, to a lactic dehydrogenase preparation in presence of lactate, cyanide and methylene blue. The effect of the nicotinamide is to prevent almost completely the breakdown of the cozymase.

Typical results with (*a*) the lactic dehydrogenase of a dialysed rabbit skeletal muscle extract and with (*b*) the lactic dehydrogenase of a ground rat kidney suspension are shown in Tables 6 and 7 respectively.

Table 6. *Effects of addition of nicotinamide aerobically*

Lactic dehydrogenase system: Rabbit skeletal muscle extract (0·5 ml.), pig heart flavoprotein (0·5 ml.), Na phosphate buffer 0·02*M* *p*H 7·4, Na lactate 0·13*M*, NaCN 0·08*M*, methylene blue 0·017 %. Cozymase (1 mg.) was incubated at 37° for 15 min. with 0·2 ml. ground rat brain suspension in phosphate buffer *p*H 7·4 in the presence and absence of nicotinamide. The mixture was then added to the lactic dehydrogenase system, and O_2 consumption measured. Air; 37°.

	μl. O_2 uptake in 1 hr.
1. Lactic dehydrogenase system with untreated cozymase	215·4
2. Lactic dehydrogenase system with cozymase previously incubated for 15 min. with 0·2 ml. brain suspension	54·1
3. Lactic dehydrogenase system with cozymase previously incubated for 15 min. with 0·2 ml. brain suspension in presence of 0·3 ml. 1 % nicotinamide	167·2
4. As in 3 but using 0·1 ml. 10 % nicotinamide	199·4
5. As in 3 but using 0·3 ml. 10 % nicotinamide	171·3
6. Lactic dehydrogenase system with untreated cozymase and with 0·3 ml. 1 % nicotinamide present (i.e. final concentration of nicotinamide =0·1 %)	195·9

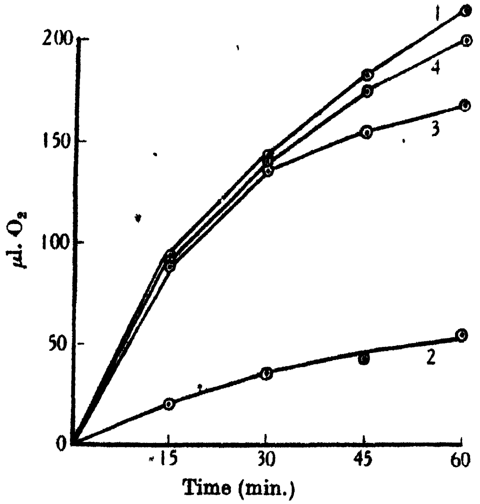


Fig. 2. Inhibitory action of nicotinamide on cozymase nucleotidase.

1. Lactic dehydrogenase system (muscle extract, etc.).
2. Lactic dehydrogenase system + 0·2 ml. ground rat brain suspension.
3. Lactic dehydrogenase system + 0·2 ml. ground rat brain suspension previously incubated with 0·1 % nicotinamide.
4. Lactic dehydrogenase system + 0·2 ml. ground rat brain suspension previously incubated with 0·3 % nicotinamide.

Examination of the results given in Table 6 and in Fig. 2 shows that the protective effect of nicotinamide increases with its concentration, but that above an optimal concentration the nicotinamide inhibits the O_2 uptake. Probably this small inhibitory effect is that already described by Baker *et al.* [1938]. This effect has not yet been explained but it seems likely that it is due to competition of nicotinamide with cozymase for lactic dehydrogenase.

The presence of nicotinamide increases the O_2 uptake of the kidney lactic dehydrogenase system, containing added cozymase even in the absence of brain suspension. This points to the presence in the ground kidney suspension of a small quantity of cozymase nucleotidase. The very powerful inhibitory effect of a brain suspension on the kidney system is almost completely neutralized by the presence of 0·1 % nicotinamide (Table 7).

Table 7. *Effects of addition of nicotinamide aerobically*

Lactic dehydrogenase system: Rat kidney suspension (prepared by grinding fresh kidney in saline, 1 g. to 10 ml., with sand and squeezing through muslin); phosphate buffer 0·02*M* *p*H 7·4; Na lactate 0·13*M*; NaCN 0·08*M*; methylene blue 0·04 %. 0·2 ml. of the kidney suspension was placed in side tube of Warburg vessel and tipped into main vessel after equilibration. Air; 37°.

	μl. O_2 uptake in 30 min.
Kidney lactic dehydrogenase system	30·1
Kidney lactic dehydrogenase system + cozymase (1 mg.)	66·0
Kidney lactic dehydrogenase system + cozymase + 0·1 % nicotinamide	89·8
Kidney lactic dehydrogenase system + cozymase (1 mg.) + 0·2 ml. ground brain suspension	10·5
Kidney lactic dehydrogenase system + cozymase (1 mg.) + 0·2 ml. ground brain suspension + 0·1 % nicotinamide	77·1

Cozymase is not only broken down by ground brain suspensions but by intact brain slices, this effect being inhibited by the presence of nicotinamide. These results are shown in Table 8. The destruction of cozymase by the intact brain slices is only partially due to cozymase nucleotidase diffusing from the tissue

Table 8. *Effect of nicotinamide on cozymase in brain slices*

Equal weights (100 mg. wet wt.) of rat brain slices were placed in Warburg vessels containing the following media:

A 0·02*M* Na phosphate *p*H 7·4, Locke solution, 2 mg. cozymase preparation.
B As *A* but containing also 0·3 % nicotinamide.
C 0·02*M* Na phosphate *p*H 7·4, Locke solution.
D As *C* but containing also 0·3 % nicotinamide.

In another vessel was placed medium *E*: 0·02*M* Na phosphate *p*H 7·4, Locke solution and 2 mg. cozymase preparation (no tissue slices present). These were incubated at 37° in O_2 for 30 min., after which slices of tissue were removed from all vessels. To *C* and *D* were then added 2 mg. cozymase preparation and these vessels were incubated for a further 30 min. in O_2 at 37°.

1 ml. of each of the above media, i.e. *A*, *B*, *C*, *D*, *E*, was then transferred to fresh Warburg vessels, containing 0·02*M* Na phosphate buffer *p*H 7·4, 0·13*M* Na lactate, 0·08*M* NaCN, 0·017 % methylene blue, and 0·16 % nicotinamide. In the side tube of each vessel was placed 0·3 ml. suspension of ground rat kidney (1 g. to 10 ml. saline). After temperature equilibration (37°) in air, the kidney suspension was tipped in the main vessels and rates of O_2 uptakes were measured.

	μl. O_2 uptake in 30 min.
Kidney lactic dehydrogenase system + 1 ml. *A*	36·4
Kidney lactic dehydrogenase system + 1 ml. *B*	112·5
Kidney lactic dehydrogenase system + 1 ml. *C*	88·7
Kidney lactic dehydrogenase system + 1 ml. *D*	131·4
Kidney lactic dehydrogenase system + 1 ml. *E*	140·4

slices in the outer medium. Much of the cozymase must be destroyed by cozymase nucleotidase within the intact tissue. The intact brain slices must, clearly, be permeable to cozymase, a conclusion already arrived at by Quastel & Wheatley [1938].

The protective effect of nicotinamide on cozymase breakdown may be demonstrated also by experiments carried out under anaerobic conditions.

If a brain suspension is, itself, used as a source of lactic dehydrogenase it may be shown that the addition of cozymase to it has but little effect on its power of reducing ferricyanide under anaerobic conditions. The addition of nicotinamide also (in the absence of added cozymase) has little or no effect on the reduction of ferricyanide. If, however, a mixture of cozymase and nicotinamide be added to the brain suspension, a powerful reduction of ferricyanide ensues. These results are shown in Tables 9 and 10. The results are easily explained by the fact that the very active cozymase nucleotidase of brain quickly destroys all free cozymase,[1] and not until the enzyme is inhibited by nicotinamide can the addition of free cozymase show its normal catalytic effect on lactic dehydrogenase activity. The results given in Table 10 show the stimulating effect of cozymase on the reducing power of brain ground in a nicotinamide Locke solution as compared with that ground in Locke solution free from nicotinamide.

Table 9. *Effects of nicotinamide anaerobically*

One rat brain (1·6 g.) was finely chopped and suspended in 16 ml. saline. 1 ml. brain suspension was placed in each Warburg vessel containing the following medium: $NaHCO_3$ 0·025*M*, NaCl 0·13*M*, Na lactate and 0·05*M* NaCN. Nicotinamide (0·3 %) or cozymase (1 mg.) or both were present in certain vessels. After removal of air by 95 % N_2 + 5 % CO_2, and equilibration at 37°, 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was tipped from side tube into main vessel.

	μl. CO_2 output in 1 hr.
Brain + lactate system	124·4
Brain + lactate system + nicotinamide	129·5
Brain + lactate system + cozymase	136·6
Brain + lactate system + cozymase + nicotinamide	341·0

Table 10. *Effects of addition of nicotinamide anaerobically*

One half (0·6 g.) of a fresh rat brain was ground in 6 ml. Locke solution. The other half of the rat brain was ground in 6 ml. Locke solution containing 3 % nicotinamide. 1 ml. of each of these suspensions was placed in a $NaHCO_3$ (0·025*M*)-Locke medium containing 0·13*M* Na lactate, 0·05*M* NaCN, with and without the addition of cozymase (1 mg.). After removal of air by 95 % N_2 + 5 % CO_2 and equilibration at 37°, 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was tipped into each vessel.

	μl. CO_2 output in 1 hr.
Brain ground in Locke solution only. No cozymase added	168·9
Brain ground in Locke solution only. Cozymase added	185·7
Brain ground in Locke solution containing nicotinamide. No cozymase added	149·6
Brain ground in Locke solution containing nicotinamide. Cozymase added	387·4

Can the apparent protective effect of nicotinamide be due to synthesis to cozymase?

A possible explanation of the effect of nicotinamide in stimulating the O_2 uptake under the given experimental conditions is that the nicotinamide is built up into free cozymase.

---

[1] We have evidence to show that brain tissue contains a small quantity of bound cozymase which is broken down to produce free cozymase on heating the tissue.

If this view is true the apparent protective effect of nicotinamide on cozymase breakdown by a brain suspension is not in fact due to a competition of nicotinamide with cozymase for a cozymase nucleotidase, but to the synthesis of cozymase by the brain suspension from nicotinamide and other constituents present in the brain suspension. It may be shown, however, that 0·3 % nicotinamide which will apparently prevent the breakdown of cozymase by a brain suspension gives rise to little or no free cozymase when incubated alone with the brain suspension. These results are shown in Table 11 and indicate that a synthesis of cozymase from nicotinamide under the experimental conditions employed does not take place. Hence the alternative explanation, that nicotinamide competes with cozymase for a cozymase nucleotidase is probably the correct one. This is supported by the result quoted in Table 10 in which it is shown that the addition of nicotinamide alone to minced brain does not increase its lactic dehydrogenase activity. Moreover, it may be shown (see Fig. 2) that the effect of nicotinamide in increasing the O_2 uptake of a lactic dehydrogenase system containing cozymase and brain suspension falls off with time, whereas if synthesis of cozymase took place, the effect of nicotinamide should increase with time.

Table 11. *Test for synthesis of cozymase*

Lactic dehydrogenase system: Washed ground pig's heart suspension (0·3 ml.); Na phosphate buffer 0·02*M* *p*H 7·4; Na lactate 0·13*M*; NaCN 0·08*M*; methylene blue 0·04 %.

Cozymase (1 mg.) or rat brain suspension or both, in the presence or absence of 0·1 ml. 10 % nicotinamide, were incubated aerobically in Warburg vessels at 37° for 15 min. The lactic dehydrogenase system was then added to each vessel and the O_2 consumption was measured. Air; 37°.

	μl. O_2 uptake in 30 min.
Lactic dehydrogenase system + cozymase	194·5
Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension	10·5
Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·3 % nicotinamide	164 5
Lactic dehydrogenase system + 0·2 ml. ground brain suspension	9·7
Lactic dehydrogenase system + 0·2 ml. ground brain suspension + 0·3 % nicotinamide	14·7

Effects of nicotinamide on succinic dehydrogenase activity in presence and absence of cozymase

It is known [Potter, 1939; Keilin & Hartree, 1940] that the addition of cozymase to a suspension of washed ground pig's heart greatly reduces the latter's succinic dehydrogenase activity. This is attributed by Keilin & Hartree to the formation of oxaloacetate from succinate by the action of succinic dehydrogenase and of malate dehydrogenase in conjunction with cozymase. The oxaloacetate formed, even in minute quantities, inhibits succinic dehydrogenase.

It may be shown that the addition of a ground brain suspension entirely removes the inhibitory effect exercised by cozymase on the succinic dehydrogenase activity of pig's heart. If nicotinamide, however, be added at the same time as the ground brain suspension, the cozymase exercises its normal inhibitory effect. These results are shown in Table 12, and are explained satisfactorily by the presence in brain of a cozymase nucleotidase which breaks down cozymase and whose activity is inhibited by the presence of nicotinamide.

It may also be shown that cozymase inhibits the anaerobic oxidation of succinate by ferricyanide in the presence of a brain suspension acting as sole source of succinic dehydrogenase. This effect is greatly increased by the addition

Table 12. *Effects of nicotinamide on succinic dehydrogenase activity in presence and absence of cozymase*

Succinic dehydrogenase system: Washed ground pig's heart suspension (1 ml.), Na succinate 0·03 *M*; phosphate buffer (0·02 *M*) *p*H 7·4. Air; 37°.

	µl. O_2 uptake in 1 hr.
Succinic dehydrogenase system	241·2
Succinic dehydrogenase system + 0·3 % nicotinamide	253·5
Succinic dehydrogenase system + cozymase (1 mg.)	119·8
Succinic dehydrogenase system + cozymase (1 mg.) + 0·3 % nicotinamide	123·9
Succinic dehydrogenase system + 0·2 ml. ground rat brain suspension	270·0
Succinic dehydrogenase system + 0·2 ml. ground rat brain suspension + 0·3 % nicotinamide	272·4
Succinic dehydrogenase system + 0·2 ml. ground rat brain suspension + cozymase (1 mg.)	236·5
Succinic dehydrogenase system + 0·2 ml. ground rat brain suspension + 0·3 % nicotinamide + cozymase (1 mg.)	130·8

to the system of nicotinamide. These results are shown in Table 13. The phenomena are explained by the presence in the brain suspension of cozymase nucleotidase. The inhibitory action of added cozymase on the succinic dehydrogenase of brain is determined by the amount of free cozymase present, and by the speed with which it can form oxaloacetic acid as compared with the speed with which it is broken down by the cozymase nucleotidase. In the presence of nicotinamide the breakdown of cozymase by the brain is inhibited and hence more free cozymase is available to build up oxaloacetic acid.

Table 13. *Effects of nicotinamide on succinic dehydrogenase activity of brain under anaerobic conditions*

Medium: $NaHCO_3$ (0·025 *M*)-saline. 95 % N_2 + 5 % CO_2; 37°. After equilibration 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was tipped into main vessel.

	µl. CO_2 output in 30 min
1 ml. ground rat brain suspension	12·0
1 ml. ground rat brain suspension + 1·0 % nicotinamide	13·7
1 ml. ground rat brain suspension + Na succinate 0·03 *M*	337·6
1 ml. ground rat brain suspension + Na succinate 0·03 *M* + 1·0 % nicotinamide	266·9
1 ml. ground rat brain suspension + Na succinate 0·03 *M* + cozymase (1 mg.)	210·9
1 ml. ground rat brain suspension + Na succinate 0·03 *M* + cozymase (1 mg.) + 1·0 % nicotinamide	128·0

Keilin & Hartree [1940] could find no inhibitory effect of cozymase on the succinic dehydrogenase of brain and attributed this apparent inactivity of cozymase to the rapid breakdown of oxaloacetic acid by brain. This explanation cannot be wholly correct as the results in Table 13 show that the presence of cozymase can inhibit succinic dehydrogenase of brain and hence that the breakdown of oxaloacetic acid by brain is not sufficiently rapid to prevent the inhibition taking place. It is more likely that the failure of Keilin & Hartree to observe an inhibitory effect of cozymase on brain succinic dehydrogenase is due to the rapid destruction of the cozymase by the brain preparation under their experimental conditions.

Effects of Indian cobra venom on lactic dehydrogenase activity in presence and absence of nicotinamide

Chain [1939] has shown that cozymase is destroyed by small quantities of cobra venom. This phenomenon we have confirmed. The inhibitory effect of the venom on the lactic dehydrogenase-cozymase system of pig heart is neutralized by the addition of nicotinamide. 0·3 % nicotinamide neutralizes almost completely the inhibitory effect of 0·5 mg. dried cobra venom. The neutralizing effect of the nicotinamide increases with increase of concentration of nicotinamide and decreases with increase of concentration of the venom. This points to the existence of an equilibrium between nicotinamide and an enzyme in the cobra venom. Whether this enzyme is identical with the cozymase nucleotidase of brain and liver remains to be seen. Results illustrating these phenomena are shown in Table 14.

Table 14. *Effects of dried Indian cobra venom on lactic dehydrogenase activity in presence and absence of nicotinamide*

Lactic dehydrogenase system: Washed ground pig's heart suspension (1 ml.); Na phosphate buffer 0·02*M* *p*H 7·4; Na lactate 0·13*M*; NaCN 0·08*M*; methylene blue 0·04 %; cozymase 1 mg. Air; 37°.

	μl. O_2 uptake in 1 hr.
Exp. 1.	
Lactic dehydrogenase system	164·8
Lactic dehydrogenase system + 0·5 mg. venom	95·2
Lactic dehydrogenase system + 0·5 mg. venom + 0·3 % nicotinamide	154·3
Lactic dehydrogenase system + 0·75 mg. venom	50·1
Lactic dehydrogenase system + 0·75 mg. venom + 0·3 % nicotinamide	99·1
Lactic dehydrogenase system + 1 mg. venom	36·2
Lactic dehydrogenase system + 1 mg. venom + 0·3 % nicotinamide	77·0
Lactic dehydrogenase system + 0·3 % nicotinamide	163·6
Exp. 2.	
Lactic dehydrogenase system	123·0
Lactic dehydrogenase system + 1 mg. venom	52·9
Lactic dehydrogenase system + 1 mg. venom + 0·03 % nicotinamide	53·0
Lactic dehydrogenase system + 1 mg. venom + 0·1 % nicotinamide	73·0
Lactic dehydrogenase system + 1 mg. venom + 0·3 % nicotinamide	92·4

Specificity of nicotinamide

Nicotinic acid will not replace nicotinamide as an inhibitor of the breakdown of cozymase by a rat brain suspension. Adenylic acid appears to have a feeble effect in protecting cozymase from breakdown. Propionamide and coramine are without action. These results are given in Table 15 and illustrate the specificity of action of nicotinamide.

Neither NaF nor $Na_4P_2O_7$ at the concentrations tried (see Table 16) prevents the breakdown of cozymase by a brain suspension.

Malate dehydrogenase and nicotinamide

Observations recorded in Table 17 show that substantially the same conclusions which apply to the lactic dehydrogenase system apply also to the malate dehydrogenase system. This is shown using the malate dehydrogenase of rat kidney under aerobic conditions and that of rat brain under anaerobic conditions. In the former case the addition of a suspension of rat brain effects an inhibition of malate oxidation which is neutralized by the presence of nicotinamide. In the latter case the presence of nicotinamide greatly increases the anaerobic oxidation of *dl*-malate in the presence of brain by ferricyanide.

Table 15. *Specificity of nicotinamide*

Lactic dehydrogenase system: 0·3 ml. ground rat kidney suspension; Na phosphate buffer 0·02M pH 7·4; Na lactate 0·13M; NaCN 0·08M; methylene blue 0·04 %. The rat kidney suspension was placed in the side tube of the vessel and tipped into the main vessel after equilibration. Air; 37°.

	μl. O_2 uptake in 1 hr.
Exp. 1.	
1. Lactic dehydrogenase system	31·7
2. Lactic dehydrogenase system + cozymase (2 mg.)	139·0
3. Lactic dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension	40·3
4. Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·3 % nicotinamide	158·6
5. Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·3 % nicotinic acid (Na salt)	41·8
6. As in 5 but using 1 % nicotinic acid (Na salt)	38·4
7. As in 5 but using 0·1 % nicotinic acid (Na salt)	47·7
8. As in 5 but using 0·03 % nicotinic acid (Na salt)	45·1
Exp. 2.	
1. Lactic dehydrogenase system	44·2
2. Lactic dehydrogenase system + cozymase (2 mg.)	257·0
3. Lactic dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension	64·6
4. Lactic dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension + 0·3 % nicotinamide	268·5
5. Lactic dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension + 10 mg. adenylic acid (muscle)	75·7
6. As in 5 but using 10 mg. yeast adenylic acid	80·9
7. Lactic dehydrogenase system + cozymase + 10 mg. adenylic acid (muscle)	208·4
8. Lactic dehydrogenase system + cozymase + 10 mg. adenylic acid (yeast)	224·0
9. Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·3 % propionamide	57·3
10. Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·1 % coramine	62·5
11. As in 10 but using 0·3 % coramine	53·4

Table 16. *Actions of fluoride and of pyrophosphate*

Lactic dehydrogenase system: Rabbit skeletal muscle extract (1 ml.), pig's heart flavoprotein, Na lactate (0·13M); NaCN (0·08M); methylene blue (0·04 %); phosphate buffer 0·02M pH 7·4 (see also Table 6). Air; 37°.

	μl. O_2 uptake in 1 hr.
Lactic dehydrogenase system + cozymase (1 mg.)	202·9
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. ground rat brain suspension	44·0
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. ground rat brain suspension in presence of 0·1M NaF	49·8
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. ground rat brain suspension in presence of 0·04M $Na_4P_2O_7$	39·2

Table 17. *Malate dehydrogenase and nicotinamide*

Aerobic

Malate dehydrogenase system: 0·3 ml. ground rat kidney suspension, Na phosphate buffer 0·02*M* *p*H 7·4; Na *dl*-malate (0·017*M*); NaCN 0·08*M*; methylene blue 0·04 %. The kidney suspension was placed in the side tube and tipped into main vessel after equilibration. Air; 37°.

	μl. O_2 uptake in 1 hr.
Malate dehydrogenase system + cozymase (2 mg.)	96·4
Malate dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension	26·6
Malate dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension + 0·3 % nicotinamide	92·5

Anaerobic

One rat brain (1·6 g.) was finely chopped and suspended in 16 ml. Locke solution. 1 ml. suspension was placed in the following medium: $NaHCO_3$ (0·025*M*)-saline, NaCN 0·05*M*, with or without Na *dl*-malate (0·017*M*), cozymase (1 mg.), and nicotinamide. After removal of air by 95 % N_2 + 5 % CO_2, and after equilibration at 37°, 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was tipped into main vessel from side tube.

	μl. CO_2 output in 1 hr.
Brain system	85·6
Brain system + 1 % nicotinamide	85·8
Brain system + *dl*-malate	111·0
Brain system + *dl*-malate + 1 % nicotinamide	198·1

DISCUSSION

It is evident that brain, both in the intact and ground condition, is able to break down cozymase at a rapid rate. The enzyme involved is present also in liver and to a small extent in kidney. It appears to be absent from a dialysed extract of rabbit skeletal muscle or from washed ground pig's heart. Possibly the same enzyme is present in cobra venom. It may also be present in tumours [cf. Boyland & Boyland, 1935] and in yeast for Leloir & Dixon [1937] have reported that crushed yeast is able to destroy cozymase.

Since brain contains a very active cozymase nucleotidase, it must be able to synthesize cozymase rapidly *in vitro* as well as *in vivo* in order to balance the nucleotidase activity and to account for the maintenance of oxidative activity of brain when this respires in lactate media. Alternatively it must be concluded that the cozymase nucleotidase of intact brain is ineffective under aerobic conditions—a conclusion which is incorrect as the foregoing observations have shown. There must therefore exist in intact brain an equilibrium between the mechanisms responsible for the synthesis of cozymase and those responsible for its breakdown. The existence of this equilibrium probably accounts, at least partially, for the fact that the respiration of damaged tissues is often much lower than that of intact tissues. In the former case damage to the tissues may impair synthetic mechanisms by alterations of cell permeability with loss of essential cell constituents by diffusion etc.

The high rates of breakdown of cozymase by brain or liver throw doubt on estimations of cozymase in tissue extracts where precautions have not been taken to destroy at once the cozymase nucleotidase. It is obviously essential that such destruction should be brought about immediately after dissection of the organ under investigation from the animal.

The presence of nicotinamide inhibits the breakdown of cozymase probably by competition with cozymase for the nucleotidase. The evidence shows that the stimulating action of nicotinamide is not due to synthesis to cozymase under the given experimental conditions.

The demonstration of the action of nicotinamide in protecting cozymase from breakdown by mammalian tissues raises the question as to whether the well-known therapeutic effects of nicotinamide may not be due partly to this action. If this view is true, substances other than nicotinamide having a similar protective effect may have similar therapeutic effects. It may be noted in this connexion that Vilter & Spies [1939] have shown that quinolinic acid will cure pellagra and that Woolley *et al.* [1938] find that a variety of nicotinic acid derivatives will cure canine black tongue. Moreover, as a result of the administration of quinolinic acid, there has been found [Vilter & Spies, 1939] an increase in concentration of cozymase in the blood. It is clear that further investigation is required of the types of molecules which will inhibit cozymase breakdown by mammalian tissues.

SUMMARY

1. The addition of a ground rat brain suspension to a lactic dehydrogenase-cozymase system or to a malic dehydrogenase-cozymase system of mammalian tissues brings about considerable diminution of the activity of the systems.

2. This is due to the destruction of cozymase by a mechanism present in brain. The evidence points to the mechanism in question being a hydrolytic enzyme which is referred to as cozymase-nucleotidase.

3. Cozymase-nucleotidase occurs in rat brain and in rat liver and to a relatively small extent in rat kidney. It is apparently absent from dialysed rabbit skeletal muscle extract and from washed ground pig's heart.

4. Cozymase nucleotidase is destroyed by heating at 70° for 5 min.

5. Evidence is given showing that cozymase is not inactivated by adsorption upon special proteins in brain.

6. The presence of nicotinamide greatly inhibits cozymase nucleotidase of brain. This may be shown by experiments carried out under aerobic and anaerobic conditions. Nicotinamide stimulates lactic and malic dehydrogenase activities of brain in presence of cozymase.

7. The stimulating effect of nicotinamide on the lactic dehydrogenase activities of tissues in presence of cozymase-nucleotidase is not due to a synthesis of nicotinamide to cozymase, but to a competition of nicotinamide with cozymase for the nucleotidase.

8. Intact brain slices break down cozymase, this effect being neutralized by the presence of nicotinamide. An equilibrium must exist in intact brain between a synthetic mechanism leading to cozymase formation and a mechanism leading to its breakdown.

9. The inhibition of succinic dehydrogenase of pig heart by cozymase is neutralized by the addition to the system of a ground brain suspension. The effect of the brain may be eliminated by the addition to the system of nicotinamide. The action of the nicotinamide here is to prevent the breakdown of the cozymase. Succinic dehydrogenase activity of a brain suspension, when examined anaerobically by the ferricyanide technique, is decreased by the addition of cozymase, the effect being enhanced by the simultaneous addition of nicotinamide. Here again the effect is due to the prevention of breakdown of cozymase by nicotinamide.

10. The action of cobra venom in inhibiting a lactic dehydrogenase-cozymase system by destruction of cozymase is retarded by nicotinamide. An equilibrium probably exists between nicotinamide and a nucleotidase in cobra venom.

11. The protective effect of nicotinamide on cozymase breakdown is not shown by nicotinic acid, coramine, propionamide, sodium fluoride or sodium pyrophosphate. Adenylic acid (of yeast or muscle) has a feeble protective action.

12. Attention is drawn to the possibility that the therapeutic effects of nicotinamide may be due partly to its protective effects on cozymase breakdown.

Our sincere thanks are due to the Rockefeller Foundation and to the Medical Research Council for their financial help, and to the Ella Sachs Plotz Foundation for a grant for the purchase of animals. We are also much indebted to Dr W. Feldberg for a supply of dried Indian cobra venom and to Dr P. Ostern for a sample of muscle adenylic acid.

REFERENCES

Baker, Fazekas & Himwich (1938). *J. biol. Chem.* **125**, 545.
Boyland & Boyland (1935). *Biochem. J.* **29**, 1097.
Chain (1939). *Biochem. J.* **33**, 407.
Corran, Green & Straub (1939). *Biochem. J.* **33**, 793.
Green, Needham & Dewan (1937). *Biochem. J.* **31**, 2329.
Keilin & Hartree (1938). *Proc. roy. Soc.* B, **125**, 171.
—— —— (1940). *Proc. roy. Soc.* B, **129**, 277.
Leloir & Dixon (1937). *Enzymologia*, **2**, 81.
Lipton & Elvehjem (1940). *J. biol. Chem.* **136**, 637.
Mann & Quastel (1941). *Nature, Lond.*, **147**, 326.
Potter (1939). *Ark. Kemi Min. Geol.* **13** B, No. 7.
Quastel & Wheatley (1938). *Biochem. J.* **32**, 936.
Straub (1939). *Biochem. J.* **33**, 787.
Vilter & Spies (1939). *Lancet*, **2**, 423.
Woolley, Strong, Madden & Elvehjem (1938). *J. biol. Chem.* **124**, 715.

53. THE SITE OF ACTION OF NARCOTICS IN RESPIRATORY PROCESSES

By M. MICHAELIS and J. H. QUASTEL

From the Biochemical Laboratory, Cardiff City Mental Hospital

(*Received 7 March 1941*)

Whilst it is well known that narcotics as a general rule inhibit respiratory processes, their effects being mostly confined to the dehydrogenases, little is known as yet of the mechanism by which narcotics, when used at the small concentrations which have biological significance, exert their inhibitory effects.

It has been shown [Quastel & Wheatley, 1932; Jowett & Quastel, 1937; Jowett, 1938] that narcotics, such as chloretone and the barbiturates, greatly reduce the oxygen uptake of brain tissue (whether in the form of a mince or of tissue slices), as well as that of other organs, when the tissue respires in media containing glucose, sodium lactate or sodium pyruvate. Definite inhibitions of oxidative processes occur at concentrations of narcotics which bring about deep narcosis, the inhibitions being reversible *in vitro* [Quastel & Wheatley, 1934; Jowett, 1938]. McClure *et al.* [1939] have demonstrated the existence of anoxia in the central nervous system during the anaesthesia brought about by barbiturates and other narcotics. The fact that glucose oxidation is quantitatively the most important respiratory process in brain *in vivo* [see Quastel, 1939] makes it likely that *in vivo*, as well as *in vitro*, narcotics exert inhibitory effects on glucose oxidation by the brain.

Narcotics do not inhibit all oxidative processes to the same extent. Urethane, for example, at concentrations which inhibit succinic dehydrogenase has no effect on xanthine oxidase [Sen, 1931]. Barbiturates, chloretone and hyoscine, at concentrations which strongly inhibit glucose oxidation in the brain, have little effect on the oxidations by the same tissue of sodium succinate or of *p*-phenylenediamine [Quastel & Wheatley, 1932].

Narcotics include a large variety of structural types, such as hydrocarbons, alcohols, ethers, urethanes, sulphones, amides etc., and it is evident that their common characteristic of inducing narcosis must depend upon certain physicochemical characters which they have in common rather than upon the possession of any special chemical structure. The well-known Overton-Meyer theory suggests one important factor in the determination of narcotic activity, i.e. the relative solubilities in lipoid and non-lipoid substances. It is evident, however, that if the nonpolar-polar character of a substance may determine, or partly determine, its entry into a cell, it gives no obvious clue to the mechanism of action of the narcotic once it is within the cell.

It is well known that many enzymic reactions taking place in cell-free extracts are inhibited by narcotics if these are present at sufficiently high concentrations. The mechanism of this inhibition is usually held to be due to a nonspecific adsorption on the enzyme surface. So far as respiratory processes are concerned, the relative lack of inhibitory action of narcotics on the indophenol (or cytochrome) oxidase has made it appear that the dehydrogenases are the most sensitive structures to narcotic inhibition.

A study [Jowett & Quastel, 1937; Jowett, 1938] of the inhibitions of respiration in intact brain tissue secured by narcotics has revealed the following facts.

(1) The inhibitions for a large class of narcotics vary little with time and are reversible, i.e. if the narcotic is washed away from the tissue the latter tends to respire again at the normal rate. Such a class includes urethane, chloral, chloretone, barbiturates such as luminal and evipan, avertin (tribromoethyl alcohol) and Mg ions. The narcotic appears to reach a mass action equilibrium with some component of the respiratory system, the inhibition being given by a simple mass action equation.

(2) The steady reversible inhibitions depend on cation activity and particularly on the potassium ion concentration of the medium bathing the brain tissue. If the potassium concentration becomes low, say one-third of that present in blood, the inhibitions increase with time, often considerably. The effect of higher potassium concentration is to stabilize the inhibition. This phenomenon would be explained if, in the presence of low concentrations of potassium ions, a factor important in influencing narcotic inhibitions diffuses from the brain slices into the outer medium.

(3) The inhibition of respiration depends on the concentration of narcotic and not upon the concentration of the substrate which is oxidized.

When, however, anaerobic oxidations accomplished by brain tissue are considered, e.g. the oxidation of lactic acid by methylene blue, it is found that the inhibition by a narcotic does depend on the substrate concentration [Davies & Quastel, 1932]. There exists an equilibrium between lactic acid and the narcotic in presence of the lactic dehydrogenase of brain, to which the mass action law is applicable. There is thus an apparent discrepancy between the two results—an equilibrium between the substrate and the narcotic under anaerobic conditions, and a lack of such equilibrium under aerobic conditions. Yet under suitable ionic conditions an equilibrium does exist under aerobic conditions between the narcotic and some component of the respiratory system.

A clue to the solution of this anomaly may be found in the fact that the low concentrations of narcotics which are highly inhibitory aerobically have but little inhibitory effect anaerobically. Thus chloretone is ten times less effective in securing an inhibition of lactic acid oxidation in presence of brain anaerobically than under aerobic conditions.

A possible explanation is that the narcotic, at the low concentrations effective under aerobic conditions, exerts its main effect on a part of the respiratory system which is inert or functionless under anaerobic conditions. That such a part of the respiratory system has the properties of an enzyme structure is evident from the fact that it is affected by surface-active narcotics of so many different chemical types. It must, however, be an enzyme with a much higher affinity for narcotics than the most sensitive enzymes—the dehydrogenases—with which we are so far familiar.

If this view is true the inhibitions of respiration of intact brain tissue obtained by low concentrations of narcotics under aerobic conditions are not due to competition of the narcotics with the substrates (e.g. lactic or pyruvic acid) for their dehydrogenases, but to the affinity of the narcotics to a special enzyme structure which plays a highly important part in the chain of reactions composing the complete aerobic respiratory process of the cell.

It is the purpose of this communication to throw some light on the site of the enzyme system which is highly sensitive to surface-active narcotics.

EXPERIMENTAL TECHNIQUE

Measurement of oxygen uptake

The Warburg respiration apparatus has been used exclusively in this investigation. The volume of fluid in the reaction vessel is always 3 ml., the *p*H being kept at 7·4 by means of sodium phosphate buffer. In anaerobic experiments sodium bicarbonate solution (0·025*M*) is used in an atmosphere of 95 % N_2+5 % CO_2. The ferricyanide technique of Quastel & Wheatley [1938] has been used in all the anaerobic work and the reader is referred to their paper for full experimental details. Experiments are usually run for 1 hr. at 37°.

Biological material

The biological material used in this work consists of thin slices of brain cortex (rat or guinea-pig), or of minced brain tissue, or of various preparations of tissue enzyme systems. The latter have been made by methods which are already fully described in the literature.

Narcotic investigated

Chloretone ($CCl_3.C(CH_3)_2OH$) is the narcotic extensively used in this investigation. This narcotic has been used because its solubility is such that it can be used conveniently in the Warburg apparatus, 0·2 ml. of a 0·5 % aqueous solution usually being placed in the side tube of the reaction vessel. This amount of chloretone on being tipped into the reaction vessel, after temperature equilibration, usually secures a large inhibition of the rate of O_2 uptake of the tissue (intact brain slices or minced brain). This drug is also used because it brings about, under suitable conditions, steady reversible inhibitions. It is also neutral in reaction, has no buffering value and is suitable for the anaerobic as well as the aerobic work. It appears to be quite stable, and dilute solutions can be kept at 0° for long periods without deterioration. It has the properties of a surface-active narcotic and has already been much investigated in this laboratory [see Jowett & Quastel, 1937; Jowett, 1938].

EFFECT OF CHLORETONE ON BRAIN RESPIRATION IN PRESENCE OF GLUCOSE

A few typical examples of the large inhibitory effects of chloretone on the respiration of brain cortex slices or of minced brain in a glucose-Locke medium are shown in Table 1. Under the conditions cited 0·033 % chloretone brings

Table 1. *Effect of chloretone on brain respiration in presence of glucose*

Exp. 1. Brain cortex slices in sodium phosphate (0·03*M*), *p*H 7·4-Locke medium (K^+= 0·004*M*; Ca^{++}=0·002*M*) containing glucose (0·016*M*); oxygen; 37°.

Animal	Chloretone concentration %	Q_{O_2}	Inhibition %
Rat	Nil	11·8	—
Rat	0·033	4·0	66
Guinea-pig	Nil	11·0	—
Guinea-pig	0·016	5·3	52

Exp. 2. Minced rat brain washed once with Locke solution and suspended in Locke solution (1 g./10 ml.); 1 ml. brain suspension in phosphate (0·03*M*) *p*H 7·4-Locke-glucose (0·016*M*) medium; air; 37°.

	µl. O_2 uptake in 1 hr.	Inhibition %
No chloretone present	181	—
With chloretone 0·033 %	120	33·7
With chloretone 0·066 %	59	67

about an inhibition of 66 % in the respiration of rat brain cortex slices and an inhibition of 33 % in the respiration of minced rat brain tissue. The respiration of brain cortex slices in a glucose medium is usually more sensitive to narcotics than that of minced tissue, a fact already noted by Jowett [1938] using ethylurethane.

EFFECT OF CHLORETONE ON ANAEROBIC GLYCOLYSIS IN PRESENCE OF BRAIN SLICES

If the action of chloretone on glucose oxidation in brain cortex slices is due to an inhibitory effect upon dehydrogenases involved in the aerobic breakdown of glucose, it may be expected that this effect will be exerted also on the dehydrogenases involved in the anaerobic breakdown of glucose. The mechanism of anaerobic breakdown of glucose in brain is not yet clear, but if the reactions involved bear some resemblance to those taking part in the anaerobic breakdown of glucose in muscle or yeast, an interplay of dehydrogenase systems will be involved. Experiments (see Table 2) show that 0·033 % chloretone exerts no inhibitory effect whatever on the anaerobic breakdown of glucose to lactic acid in presence of rat brain cortex slices. The result shows that if a narcotic-sensitive dehydrogenase system plays an important role in the aerobic breakdown of glucose, it is either absent from, or is without influence in, the reactions involved in the anaerobic breakdown of glucose by brain.

Table 2. *Effect of chloretone on anaerobic glycolysis by brain*

Rat brain cortex slices in sodium bicarbonate (0·025*M*)-Locke solution containing 0·025*M* glucose and 0·003*M* sodium pyruvate; 95 % N_2 + 5 % CO_2; 37°.

		$Q_M^{N_2}$	
Medium without added chloretone	15·2	19·8	16·5
Medium with chloretone 0·033 %	15·1	—	—
Medium with chloretone 0·05 %	—	17·8	16·4

ANAEROBIC OXIDATION OF GLUCOSE BY BRAIN TISSUE

A more direct approach to the problem as to whether a narcotic exerts large inhibitory effects on dehydrogenases involved in glucose breakdown was made in the following manner.

In the ferricyanide technique of Quastel & Wheatley [1938] use is made of the fact that in the reduction of 1 mol. ferricyanide 1 mol. acid is formed and in bicarbonate media this gives rise to 1 mol. CO_2 which is estimated manometrically. The relevant equations are:

$$H + Fe(CN)_6''' \rightarrow H^+ + Fe(CN)_6''''$$
$$H^+ + HCO_3' \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O.$$

The technique has been used for the study of cozymase-linked systems (since ferricyanide readily oxidizes reduced cozymase [Haas, 1937]), as well as for the study of other dehydrogenase systems (e.g. those involving succinic acid or choline) which do not depend for their activity on cozymase. The technique can be used in the investigations of anaerobic oxidations brought about by intact tissue slices as well as by minced tissue or by tissue extracts. Full experimental details may be found in the paper quoted.

The addition of ferricyanide solution containing a little bicarbonate (1 ml. 0·16*M* $NaHCO_3$ to 5 ml. 10 % $Na_3Fe(CN)_6$) to minced brain tissue in a bicarbonate-glucose medium in an atmosphere of 95 % N_2+5 % CO_2 results in but little evolution of CO_2 above that obtained, by glycolysis, in the absence of ferricyanide. The addition of pyocyanine to the medium, followed by that of ferricyanide, brings about a very large increase in the velocity of CO_2 output. Pyocyanine is reduced in the presence of brain tissue by glucose, or by a breakdown product of glucose, and the reduced pyocyanine is oxidized directly by ferricyanide. Pyocyanine in this way acts as a catalyst in the anaerobic oxidation of glucose (or of a breakdown product of glucose) by ferricyanide. A typical result with minced rat brain tissue is shown in Table 3. In this case a CO_2 output of 144·5 μl. in the presence of glucose and ferricyanide is increased to 520·2 μl. by the addition of pyocyanine.

The same phenomenon is observed with brain cortex slices. A typical result is given in Table 4. The addition of pyocyanine increases the $Q^{N_2}_{CO_2}$ (μl. CO_2 produced per hr. per mg. dry weight of tissue) from 17·8 to 37·8.

Table 3. *Anaerobic oxidation of glucose, lactate and pyruvate by minced brain*

Minced brain tissue washed with saline and suspended in saline; 1 ml. suspension in bicarbonate (0·025 *M*)-saline; glucose, 0·016 *M*; sodium lactate, 0·02 *M*; sodium pyruvate, 0·015 *M*; pyocyanine chloride, 1·6 mg. per 100 ml. 0·2 ml. 8·3 % sodium ferricyanide solution added where indicated; 95 % N_2 + 5 % CO_2; 37°.

	μl. CO_2 output		
Substrates etc. added	Rat 0·1 g. wet wt. tissue (1 hr.)	Rat 0·15 g. wet wt. tissue (1 hr.)	Guinea-pig 0·2 g. wet wt. tissue (2 hr.)
Ferricyanide	45·0	86·8	51·5
Pyocyanine + ferricyanide	175·5*	113·3	61·7
Glucose	105·0	—	—
Glucose + ferricyanide	144·5	—	—
Lactate + ferricyanide	—	107·2	—
Pyruvate + ferricyanide	—	—	68·4
Glucose + pyocyanine + ferricyanide	520·2*	—	—
Lactate + pyocyanine + ferricyanide	—	536·4	—
Pyruvate + pyocyanine + ferricyanide	—	—	248·0

* Pyocyanine chloride = 3·2 mg. per 100 ml.

Table 4. *Effect of chloretone on anaerobic oxidation of glucose by brain*

Rat brain cortex slices in bicarbonate (0·025 *M*)-Locke medium; 0·2 ml. 8·3 % sodium ferricyanide solution added where indicated; pyocyanine chloride, 3·2 mg. per 100 ml.; glucose, 0·01 *M*; 95 % N_2 + 5 % CO_2; 37°.

	$Q^{N_2}_{CO_2}$	
Glucose + ferricyanide	17·8	
Pyocyanine + ferricyanide	8·5	
Glucose + pyocyanine	12·1	13·7
Glucose + pyocyanine + ferricyanide	37·8	31·2
Glucose + pyocyanine + ferricyanide + chloretone (0·083 %)	29·5	26·8
Inhibition by chloretone	21·9 %	14·1 %

EFFECT OF CHLORETONE ON THE ANAEROBIC OXIDATION OF GLUCOSE BY BRAIN CORTEX SLICES

The presence of small concentrations of chloretone which inhibit vigorously the aerobic oxidation of glucose has no effect on the anaerobic oxidation of glucose by brain tissue in the presence of pyocyanine and ferricyanide. Even so high a concentration as 0·083 % chloretone, which inhibits the O_2 uptake of rat brain cortex slices in a glucose medium by over 80 %, only inhibits the anaerobic oxidation of glucose by pyocyanine and ferricyanide by about 20 %. Typical results are noted in Table 4. This result indicates the relative insensitivity to chloretone of the dehydrogenase system responsible for the reduction of pyocyanine by brain tissue in presence of glucose.

It is possible that the substance which brings about the reduction of pyocyanine in the experiment just quoted is lactic acid, produced from glucose by glycolysis, and that the dehydrogenase system involved is that mainly concerned with the oxidation of lactic acid.

The effects of chloretone on the aerobic and anaerobic oxidations of lactic acid in the presence of brain will, therefore, now be considered.

EFFECTS OF CHLORETONE ON THE AEROBIC AND ANAEROBIC OXIDATIONS OF LACTIC ACID

As with the oxidation of glucose, narcotics exercise a large inhibition on the aerobic oxidation of lactic acid in the presence of brain. A few typical results, obtained both with brain cortex slices and minced brain tissue, are shown in Table 5. The inhibition by chloretone is of the same order of magnitude as with glucose oxidation.

Table 5

I. Guinea-pig brain cortex slices in Na phosphate (0·03 *M*) *p*H 7·4-Locke solution; sodium lactate, 0·02 *M*; O_2; 37°.

	Q_{O_2}	
Lactate	13·4	16·5
Lactate + chloretone 0·016 %	6·9	7·5
Inhibition by chloretone	49 %	54 %

II. Minced washed rat brain suspension (1 g. to 10 ml.) in Na phosphate (0·03 *M*) *p*H 7·4-saline; 1 ml. suspension taken; sodium lactate, 0·01 *M*; air; 37°.

	μl. O_2 uptake in 1 hr.	
Lactate	249·1	194
Lactate + chloretone 0·083 %	60·0	—
Lactate + chloretone 0·033 %	—	129

Pyocyanine greatly accelerates the anaerobic oxidation by ferricyanide of lactic acid in presence of brain tissue, as may be seen by a glance at Table 3. Chloretone, however, even at the concentration of 0·083 % exerts no inhibitory effect on the anaerobic oxidation of lactic acid by pyocyanine and ferricyanide in presence of rat brain cortex slices (see Table 6).

Table 6. *Effect of chloretone on anaerobic oxidation of lactate*

Rat brain cortex slices in bicarbonate (0·025 *M*)-Locke medium; 0·2 ml. 8·3 % Na ferricyanide solution added; sodium lactate, 0·016 *M*; pyocyanine chloride, 3·2 mg. per 100 ml.; 95 % N_2 + 5 % CO_2; 37°.

	$Q_{CO_2}^{N_2}$
Pyocyanine + ferricyanide + lactate	20·8
Pyocyanine + ferricyanide + lactate + chloretone 0·083 %	19·6

If, therefore, the lactic dehydrogenase system responsible for the reduction of pyocyanine by lactic acid is identical with that which brings about the activation of lactic acid under aerobic conditions, the latter cannot be the narcotic-sensitive component of the aerobic respiratory mechanism.

EFFECT OF CHLORETONE ON THE AEROBIC AND ANAEROBIC OXIDATIONS OF PYRUVIC ACID BY BRAIN TISSUE

Pyruvic acid oxidation by brain, either in the form of cortex slices or of a mince, is as sensitive to the action of chloretone as the oxidation of glucose or of lactic acid. Typical results are shown in Table 7, in which it is also shown that the addition of pyocyanine under aerobic conditions has no appreciable effect upon the chloretone inhibition.

Table 7

I. Rat brain cortex slices in Na phosphate (0·03 *M*, *p*H 7·4)-Locke-Na pyruvate (0·016 *M*) medium; O_2; 37°.

	Q_{O_2}
No chloretone present	10·1
With chloretone 0·033 %	3·5
Inhibition by chloretone	66 %

II. Minced guinea-pig brain washed thrice with saline and suspended in saline (1 g. to 5 ml.); 1 ml. suspension in Na phosphate (0·03 *M*, *p*H 7·4)-saline; Na pyruvate, 0·015 *M*; pyocyanine chloride, 1·6 mg. per 100 ml.; air; 37°.

Substrates etc. added	μl. O_2 uptake in 1 hr.
Nil	30·1
Pyocyanine	36·6
Na pyruvate	105·3
Na pyruvate + pyocyanine	99·1
Na pyruvate + chloretone 0·083 %	6·9 (inhibition = 93 %)
Na pyruvate + pyocyanine + chloretone 0·083 %	10·8 (inhibition = 89 %)

Anaerobically, pyruvic acid is but feebly attacked by ferricyanide in presence of brain cortex slices. The addition of pyocyanine greatly accelerates the rate of oxidation by ferricyanide, a $Q_{CO_2}^{N_2}$ of 6·6 in the absence of pyocyanine being increased to 17·9 in its presence. The addition of a relatively high concentration of chloretone has but little inhibitory effect upon the anaerobic oxidation of pyruvic acid by rat brain cortex slices. A representative result is shown in Table 8.

Table 8. *Effect of chloretone on anaerobic oxidation of pyruvate*

Rat brain cortex slices in bicarbonate (0·025 *M*)-Locke medium; 0·2 ml. 8·3 % Na ferricyanide added where indicated; pyocyanine chloride, 3·2 mg. per 100 ml.; Na pyruvate, 0·016 *M*; 95 % N_2 + 5 % CO_2; 37°.

	$Q_{CO_2}^{N_2}$
Ferricyanide	4·1
Pyocyanine + ferricyanide	8·5
Pyruvate + ferricyanide	6·6
Pyruvate + pyocyanine + ferricyanide	17·9
Pyruvate + pyocyanine + ferricyanide + chloretone (0·083 %)	13·1
Inhibition by chloretone	26·8 %

It follows from these results that if a pyruvic dehydrogenase is common to both the aerobic and anaerobic oxidations of pyruvic acid in brain, this enzyme cannot be regarded as the narcotic-sensitive component of the aerobic system.

EFFECT OF CHLORETONE ON THE ANAEROBIC OXIDATION OF LACTIC ACID IN PRESENCE OF CRUSHED YEAST

Lactic acid is vigorously oxidized in presence of a suspension of dialysed crushed baker's yeast by ferricyanide under anaerobic conditions in the absence of any added carrier such as pyocyanine. The crushing of the yeast was accomplished in a wet-crushing mill [Booth & Green, 1938] and the crushed yeast was dialysed against distilled water overnight in the ice chest.

The addition of potassium ferricyanide solution to a suspension of the crushed yeast in a bicarbonate medium results in an evolution of CO_2, due to the oxidation of substances still present in the suspension. The further addition of sodium lactate results in a large acceleration of the rate of CO_2 output. It will be seen from Table 9 that an evolution of 210 μl. CO_2 in 10 min. was increased, by the presence of lactate, to 683·5 μl. CO_2 in the same time.

Oxidation of lactic acid, in presence of crushed yeast, by ferricyanide occurs also on addition of sodium cyanide. The rate is not materially affected by the addition of a cozymase preparation (a slight increase in the rate due to added cozymase is noted in Table 9).

Table 9

Baker's yeast (40 g. yeast and 20 ml. water) crushed in mill and dialysed against distilled water; 1 ml. suspension in bicarbonate (0·025*M*)-saline; 0·2 ml. 8·3 % K ferricyanide solution added; 95 % N_2 + 5 % CO_2; 37°.

	Substances added	μl. CO_2 output in 10 min.
I.	Ferricyanide	210·0
	Ferricyanide + chloretone (0·083 %)	200·1
	Na lactate (0·015*M*) + ferricyanide	683·5
	Na lactate (0·015*M*) + ferricyanide + chloretone (0·083 %)	604·9
		μl. CO_2 output in 30 min.
II.	NaCN (0·03*M*) + ferricyanide	86·0
	Na lactate (0·01*M*) + NaCN (0·03*M*) + ferricyanide	638·3
	Na lactate (0·01*M*) + NaCN (0·03*M*) + 1 mg. cozymase + ferricyanide	705·6

The addition of chloretone (0·083 %) depresses only slightly the rate of oxidation of lactic acid by ferricyanide in presence of yeast lactic dehydrogenase (Table 9).

It is evident, therefore, that yeast lactic dehydrogenase, like that of brain, is not highly sensitive to the narcotic.

EFFECT OF CHLORETONE ON MUSCLE DEHYDROGENASE

A preparation of a dialysed extract of rabbit skeletal muscle was used as a source of muscle dehydrogenase. It was made according to the method of Green *et al.* [1937]. As shown by Quastel & Wheatley [1938], this extract contains dehydrogenases which, in the presence of cozymase and the appropriate substrates, rapidly reduce ferricyanide under anaerobic conditions.

Sodium hexosediphosphate and sodium α-glycerophosphate are examples of substrates which are oxidized rapidly by ferricyanide in the presence of rabbit skeletal muscle extract containing cozymase.

Chloretone (0·083 %) does not inhibit the anaerobic oxidation of hexosediphosphate by this system.

The oxidation of α-glycerophosphate is inhibited by chloretone (0·033 %) to the extent of 10 %, the inhibition being increased to 30 % when this concentration of chloretone is doubled. These facts are noted in Table 10.

It seems, therefore, that although the α-glycerophosphate dehydrogenase system of skeletal muscle shows some sensitivity to the narcotic, it is not nearly as sensitive as the aerobic respiratory systems of intact brain tissue in glucose media.

Table 10

Rabbit skeletal muscle extract dialysed; 1 ml. extract in 0·025 M bicarbonate-saline; 0·2 ml. 8·3 % K ferricyanide added where indicated; 1 mg. cozymase preparation added; Na hexosediphosphate, 0·23 %; Na α-glycerophosphate, 0·02 M; 95 % N_2 + 5 % CO_2; 37°.

	μl. CO_2 output in 15 min.
I. Na hexosediphosphate + cozymase	65·1
Na hexosediphosphate + cozymase + ferricyanide	409·2
Na hexosediphosphate + cozymase + ferricyanide + chloretone (0·083 %)	459·0

	μl. CO_2 output in 1 hr.
II. Na α-glycerophosphate + cozymase + ferricyanide	466·4
Na α-glycerophosphate + cozymase + ferricyanide + chloretone (0·016 %)	436·3
Na α-glycerophosphate + cozymase + ferricyanide + chloretone (0·033 %)	416·3
Na α-glycerophosphate + cozymase + ferricyanide + chloretone (0·05 %)	411·0
Na α-glycerophosphate + cozymase + ferricyanide + chloretone (0·066 %)	323·4

EFFECTS OF CHLORETONE ON AN OXIDIZING SYSTEM COMPOSED OF RABBIT SKELETAL MUSCLE EXTRACT AND CRUSHED BAKER'S YEAST

The oxygen uptake obtained when sodium α-glycerophosphate or sodium hexosediphosphate is incubated at 37° with dialysed crushed baker's yeast is much increased when dialysed rabbit skeletal muscle extract is added to the mixture. This takes place although the incubation of sodium α-glycerophosphate or hexosediphosphate with the muscle extract alone leads to no consumption of oxygen. The increased oxygen uptake is doubtless due to the fact that the muscle extract supplies increased amounts of dehydrogenases, which in presence of cozymase and the cytochrome system provided by the crushed yeast, lead to oxidation of the α-glycerophosphate and nexosediphosphate. Typical results are shown in Table 11 (A and B) and Table 12 (A).

Table 11. *Rabbit skeletal muscle extract and crushed baker's yeast (20 %)*

A. 1 ml. dialysed muscle extract, 0·5 ml. crushed yeast in Na phosphate (0·03 M), pH 7·4-saline; Na α-glycerophosphate, 0·02 M; air; 37°.

	μl. O_2 uptake in 1 hr.
Muscle extract + α-glycerophosphate	0·0
Crushed yeast + α-glycerophosphate	89·3
Muscle extract + crushed yeast + α-glycerophosphate	198·5

B. 1·5 ml. dialysed muscle extract and 0·2 ml. dialysed crushed yeast in Na phosphate, pH 7·4-saline; Na α-glycerophosphate, 0·02 M air; 37°.

	μl. O_2 uptake in 1 hr.
Crushed yeast + α-glycerophosphate	66·4
Crushed yeast + α-glycerophosphate + muscle extract	188·3
Crushed yeast + α-glycerophosphate + muscle extract + chloretone (0·05 %)	89·3
Inhibition by chloretone	51·7 %

C. 1·5 ml. dialysed muscle extract and 0·2 ml. dialysed crushed yeast in bicarbonate (0·025 M)-saline; Na α-glycerophosphate, 0·02 M; 0·2 ml. 8·3 % K ferricyanide; 95 % N_2 + 5 % CO_2; 37°.

	μl. CO_2 output in 1 hr.
Ferricyanide + muscle extract + crushed yeast + α-glycerophosphate	343·8
Ferricyanide + muscle extract + crushed yeast + α-glycerophosphate + chloretone (0·05 %)	260·4
Inhibition by chloretone	24·3 %

Table 12. *Rabbit skeletal muscle extract and crushed baker's yeast*

A. 1·5 ml. dialysed rabbit muscle extract and 0·2 ml. dialysed crushed baker's yeast in Na phosphate (0·03 *M*), *p*H 7·4-saline; Na hexosediphosphate, 0·005 *M*; air; 37°.

	μl. O_2 uptake in 1 hr.
Crushed yeast + hexosediphosphate	37·3
Crushed yeast + hexosediphosphate + muscle extract	129·9
Crushed yeast + hexosediphosphate + muscle extract + chloretone (0·05 %)	60·4
Inhibition by chloretone	53·4 %

B. 1·5 ml. dialysed rabbit muscle extract and 0·2 ml. dialysed crushed baker's yeast in Na bicarbonate (0·025 *M*)-saline; Na hexosediphosphate, 0·005 *M*; 0·2 ml. 8·3 % K ferricyanide; 95 % N_2 + 5 % CO_2; 37°.

	μl. CO_2 output in 1 hr.
Crushed yeast + muscle extract + hexosediphosphate + ferricyanide	628·9
Crushed yeast + muscle extract + hexosediphosphate + ferricyanide + chloretone (0·05 %)	620·1
Inhibition by chloretone	1·2 %

The addition of chloretone (0·05 %) to the system—skeletal muscle extract and dialysed crushed yeast—results in over 50 % inhibition of the oxygen uptake obtained in the presence of either sodium α-glycerophosphate (Table 11 B) or of sodium hexosediphosphate (Table 12).

The system of muscle extract and crushed yeast can be investigated anaerobically using the ferricyanide technique. Under such circumstances the cytochrome system presumably plays no part, and the reduction of ferricyanide will be accomplished through the activities of the combined dehydrogenases of the muscle extract and crushed yeast, the cozymase necessary for the reduction being supplied largely by the yeast. Investigation of this system shows that the addition of chloretone (0·05 %) brings about little or no inhibition of the anaerobic oxidation of hexosediphosphate (Table 12 B) and 24 % inhibition of the rate of anaerobic oxidation of α-glycerophosphate (Table 11 C).

These results show that whilst the oxygen uptake of a respiratory system—muscle extract, crushed yeast and substrate—is considerably inhibited by a narcotic, the amount of inhibition by the same concentration of narcotic is greatly decreased when the system is examined anaerobically. Since the dehydrogenases therefore cannot be the narcotic-sensitive component of the respiratory system, it follows that the narcotic must act vigorously on some component occupying an intermediate position between the dehydrogenase and the cytochrome systems.

EFFECT OF CHLORETONE ON AN OXIDIZING SYSTEM COMPOSED OF RABBIT SKELETAL MUSCLE EXTRACT, COZYMASE AND CYTOCHROME OXIDASE

Substantially the same results as those just recorded are obtained if crushed yeast be replaced by a mixture of cozymase and cytochrome oxidase, prepared from pig heart according to the method of Keilin & Hartree [1938]. A typical result given in Table 13A shows that oxygen consumption takes place only in the complete system—cytochrome oxidase, muscle extract, sodium α-glycerophosphate and cozymase. The cytochrome and flavoprotein necessary for the system are present in the cytochrome oxidase preparation.

Addition of chloretone (0·083 %) to this system brings about over 80 % inhibition of the rate of oxygen uptake (Table 13 B). Thus a relatively narcotic-insensitive system—muscle extract, α-glycerophosphate, cozymase—is made narcotic-sensitive by the addition to it of a cytochrome oxidase preparation.

Table 13. *Rabbit muscle extract and pig heart muscle (cytochrome oxidase)*

1 ml. dialysed rabbit muscle extract and 0·5 ml. cytochrome oxidase preparation in Na phosphate (0·03 M) pH 7·4-saline; 1 mg. cozymase preparation; Na α-glycerophosphate, 0·02 M; air; 37°.

		µl. O_2 uptake in 1 hr.
A.	Cytochrome oxidase preparation + muscle extract + α-glycerophosphate	0·0
	Cytochrome oxidase preparation + cozymase + α-glycerophosphate	0·0
	Cytochrome oxidase preparation + muscle extract + α-glycerophosphate + cozymase	96·1
B.	Cytochrome oxidase preparation + muscle extract + α-glycerophosphate + cozymase	136·0
	Cytochrome oxidase preparation + muscle extract + α-glycerophosphate + cozymase + chloretone (0·083 %)	16·7
	Inhibition by chloretone	87 %

EFFECT OF CHLORETONE ON AN OXIDIZING SYSTEM COMPOSED OF GLUCOSE DEHYDROGENASE, COZYMASE AND CYTOCHROME OXIDASE

A water-soluble preparation of glucose dehydrogenase was made by acetone treatment of pig liver according to the method of Harrison [1931]. This preparation, as shown by Hawthorne & Harrison [1939], in presence of cozymase, cytochrome, cytochrome oxidase and glucose (probably with the co-operation of a flavoprotein) takes up oxygen, the glucose being oxidized to gluconic acid. Only the complete system takes up oxygen as shown by the results given in Table 14. Cytochrome was, in fact, not added in the experiment recorded in Table 14, as the cytochrome oxidase preparation contained sufficient quantities of it.

Table 14. *Glucose dehydrogenase (acetone pig liver) preparation and cytochrome oxidase (pig heart) preparation*

I. 1 ml. glucose dehydrogenase preparation (0·25 g. in 8 ml. saline), 0·5 ml. cytochrome oxidase preparation, 1 mg. cozymase preparation in Na phosphate (0·03 M), pH 7·4-saline; glucose, 0·016 M; air; 37°.

		µl. O_2 in 1 hr.
	Glucose dehydrogenase + cozymase + glucose	0·0
	Glucose dehydrogenase + cytochrome oxidase + glucose	6·0
	Glucose dehydrogenase + cytochrome oxidase + cozymase	5·0
	Glucose dehydrogenase + cytochrome oxidase + cozymase + glucose	80·6
II.	Glucose dehydrogenase (1 ml. of solution of 0·5 g. in 8 ml. saline) + cytochrome oxidase + cozymase + glucose (0·1 M)	341·2
	Glucose dehydrogenase + cytochrome oxidase + cozymase + glucose + chloretone (0·083 %)	8·4
	Inhibition by chloretone	97 %

The complete system—glucose dehydrogenase, cytochrome oxidase, cozymase and glucose—is highly sensitive to chloretone. This narcotic at a concentration of 0·083 % brings about over 90 % inhibition of the rate of oxygen uptake (Table 14).

The system may be investigated anaerobically by the ferricyanide technique. Results in Table 15 (I) show that the addition of cozymase greatly increases the rate of CO_2 output obtained by incubating anaerobically the glucose dehydrogenase preparation with glucose and ferricyanide. The system is thus a typical cozymase-linked dehydrogenase system.

The addition of chloretone at a concentration of 0·083 % secures no inhibition within experimental error of the rate of anaerobic oxidation of glucose by ferricyanide in presence of glucose dehydrogenase and cozymase (Table 15).

Table 15. *Glucose dehydrogenase (acetone pig liver) preparation*

I. 1 ml. glucose dehydrogenase preparation; 1 mg. cozymase preparation; 0·025*M* Na bicarbonate-saline; glucose, 0·016*M*; 0·2 ml. 8·3 % K ferricyanide solution added; 95 % N_2 + 5 % CO_2; 37°.

	μl. CO_2 output in 1 hr.
Glucose dehydrogenase	88·9
Glucose dehydrogenase + cozymase	96·0
Glucose dehydrogenase + glucose	68·5
Glucose dehydrogenase + cozymase + glucose	233·3

II. 1 ml. glucose dehydrogenase preparation; 1 mg. cozymase preparation; 0·025*M* Na bicarbonate-saline; glucose, 0·1*M*; 0·2 ml. 8·3 % K ferricyanide added; 95 % N_2 + 5 % CO_2; 37°.

	μl. CO_2 output in 30 min.
Glucose dehydrogenase + cozymase	75·4
Glucose dehydrogenase + cozymase + chloretone (0·083 %)	75·6
Glucose dehydrogenase + cozymase + glucose	335·8
Glucose dehydrogenase + cozymase + glucose + chloretone (0·083 %)	329·7
Inhibition by chloretone	2 %

The conclusion must be drawn that, as with the α-glycerophosphate system of muscle, the glucose dehydrogenase of liver is relatively narcotic-insensitive, and that the glucose-oxidizing system becomes highly narcotic-sensitive on the addition to it of a cytochrome oxidase preparation.

CHLORETONE AND CYTOCHROME OXIDASE

It is well known that cytochrome oxidase is relatively insensitive to narcotics. Quastel & Wheatley [1932] for example showed that chloretone and barbiturates, at concentrations which greatly inhibited the respiration of brain in glucose media, have little or no effect upon the oxidation of *p*-phenylenediamine or of Na succinate by brain tissue. It must be concluded that the narcotic inhibits neither the mechanism by which oxygen enters the cell nor that by which it is brought into a state of activity.

CHLORETONE AND THE AEROBIC RESPIRATORY SYSTEM

The observations which have been recorded so far may be summarized as follows:

1. Chloretone, at the small concentrations which inhibit the respiration of intact brain tissue, has but little inhibitory effect upon the dehydrogenase systems which may be expected to play a part in respiration. This insensitivity of the known dehydrogenases towards the small but biologically important concentrations of narcotic probably accounts for the fact that the inhibition of respiration by a narcotic is independent of the concentration of the substance burned [Jowett & Quastel, 1937].
2. Chloretone has little or no inhibitory effect on the activity of cytochrome oxidase.
3. A respiratory system composed of a mixture of a dehydrogenase system and a cytochrome oxidase preparation—both of which components are separately relatively narcotic-insensitive—is narcotic-sensitive.

The conclusion to be drawn from these observations is that the component in the complete respiratory system which is highly narcotic-sensitive must be a factor playing an intermediate role between the dehydrogenase and cytochrome oxidase systems. This factor must be lacking in the respiratory system involving the aerobic oxidation of succinate in animal tissues, since this is relatively narcotic-insensitive.

The narcotic-sensitive factor in the system involving the oxidation of lactic acid by animal tissues must take part in the reactions between reduced cozymase and cytochrome (or one of the cytochromes). A number of substances may be involved in this intermediate section of the respiratory system and these are not yet clearly defined or identified.

The evidence points, however, to the fact that a flavoprotein plays an important role in this part of the respiratory system. A flavoprotein (diaphorase) is known to be reduced by reduced cozymase and there is some evidence that reduced flavoproteins may react with components of the cytochrome system [Theorell, 1936; Dewan & Green, 1938]. Haas *et al.* [1940] have reported the existence of a flavoprotein in yeast which completes the oxidation-reduction chain between hexosemonophosphate and cytochrome *c*.

Flavoprotein, together with the cytochrome components, is present in the cytochrome oxidase preparation used in the experiments of which the results are given in Tables 13 and 14.

An investigation, therefore, was made of the effects of chloretone on the accelerating action of flavoprotein in an oxidizing system.

CHLORETONE AND FLAVOPROTEIN

Heart muscle flavoprotein was prepared by the method of Straub [1939]. Its catalytic activity in oxidation has been demonstrated by Corran *et al.* [1939]. As shown by these authors the oxygen uptake of a system composed of rabbit skeletal muscle extract, cozymase, sodium lactate, sodium cyanide and methylene blue is much increased by the addition of flavoprotein. A typical result is shown in Table 16 (I). The flavoprotein is reduced by reduced cozymase, and methylene blue is in turn reduced by the reduced flavoprotein. The reduced methylene blue is autoxidizable, and hence a chain of reactions securing the oxidation of lactate is formed. Cyanide is added to the system to fix the pyruvate, which would otherwise inhibit the activity of the lactic dehydrogenase of the muscle extract.

Table 16

Constituents present in Na phosphate (0·02 *M*) *p*H 7·4-saline medium: 0·3 ml. dialysed rabbit skeletal muscle extract, 0·5 ml. flavoprotein preparation, 1 mg. cozymase preparation, Na cyanide (0·083 *M*), Na lactate (0·13 *M*) and methylene blue (0·017 %); air; 37°.

		µl. O_2 uptake in 1 hr.
I.	Muscle extract + cozymase + Na lactate + NaCN + methylene blue	62·5
	Flavoprotein + cozymase + Na lactate + NaCN + methylene blue	44·2
	Muscle extract + flavoprotein + cozymase + Na lactate + NaCN + methylene blue	303·2
II.	Muscle extract + flavoprotein + cozymase + Na lactate + NaCN + methylene blue	268·5
	Muscle extract + flavoprotein + cozymase + Na lactate + NaCN + methylene blue + chloretone (0·083 %)	270·5
	% inhibition by chloretone	Nil

Addition of chloretone at a relatively high concentration (0·083 %) has no inhibitory effect on this system (Table 16 (II)).

It follows from this fact that heart flavoprotein is not itself unduly affected by the narcotic. The narcotic appears to affect neither the reduction of flavoprotein by cozymase nor the reduction of methylene blue by reduced flavoprotein. Yet in a respiratory system consisting of rabbit skeletal muscle extract, cozymase, flavoprotein, cytochrome oxidase, sodium lactate and semicarbazide (added to fix the pyruvate) the addition of chloretone (0·083 %) causes 52% inhibition of the velocity of oxygen uptake. This fact is noted in Table 17.

Table 17

Constituents present: 1 ml. dialysed rabbit skeletal muscle extract; 1 ml. flavoprotein preparation, 1 mg. cozymase preparation, 0·2 ml. cytochrome oxidase preparation, semicarbazide 0·03 *M*, Na lactate 0·13 *M* and saline; air; 37°.

	μl. O_2 uptake in 1 hr.
Muscle extract + cozymase + flavoprotein + cytochrome oxidase + semicarbazide + Na lactate	135·1
Muscle extract + cozymase + flavoprotein + cytochrome oxidase + semicarbazide + Na lactate + chloretone (0·083 %)	64·8
Inhibition by chloretone	52 %

Unless, therefore, there is still another enzyme which is highly narcotic-sensitive and which is playing a role between flavoprotein and one or more of the cytochrome components, it must be concluded that either

(*a*) There is present in animal tissues a special narcotic-sensitive flavoprotein which forms an important link between reduced cozymase and the cytochrome system. It should be noted that the cytochrome *c* reductase of Haas *et al.* [1940] is a flavoprotein composed of a specific protein and an alloxazine mononucleotide. It is very unstable compared with other flavoproteins and its activity is destroyed by acetone and dioxane. Conceivably such a flavoprotein may be highly narcotic-sensitive; or

(*b*) Some component of the cytochrome system itself is highly narcotic-sensitive (see e.g. Keilin & Hartree [1939]). This component would obviously play little part in the oxidation of either sodium succinate or of *p*-phenylenediamine in animal tissues.

The oxidation of pyruvate by tissues is greatly inhibited by a narcotic such as chloretone (Table 7) and this system is presumably independent of the action of cozymase. There is some evidence, however [Lipmann, 1939], that a flavoprotein is involved in pyruvate oxidation by bacteria and conceivably a flavoprotein is involved in the oxidation of pyruvate by animal tissues.

The oxidation of *dl*-alanine by rat kidney slices has been shown [Jowett & Quastel, 1937] to be inhibited by small concentrations of a narcotic. Since amino-acid oxidase has been identified as a flavoprotein, it is possible, from what has been said, that the narcotic acts here on the process intermediate between the flavoprotein and a cytochrome component.

With our present state of incomplete knowledge of the components of respiratory systems of animal tissues it would be unwise to speculate further. A solution of the problem of the precise site at which a narcotic acts, in biologically important concentrations, in respiratory systems, depends on a more complete elucidation of the details of these systems. It may be suggested, however, that the property of narcotics of inducing, at low concentrations, strong inhibition of respiratory activity may be used more extensively than hitherto in the investigation of the components of respiratory systems.

What is clear from these results is that, in the inhibition of respiratory activity effected by low concentrations of narcotics, the familiar dehydrogenases, which have been popularly supposed to be the narcotic-sensitive components of the respiratory processes, are practically unaffected. The narcotic seems to act at a site involving a special flavoprotein or an intermediate between flavoprotein and the cytochrome oxidase system.

SUMMARY

1. The effects of chloretone on the oxidations of glucose, sodium lactate and sodium pyruvate in presence of brain tissue (cortex slices and minced brain), both under aerobic and anaerobic conditions have been investigated. Concentrations of chloretone which effect large inhibitions of the aerobic oxidations have but little effect upon the anaerobic oxidations (using the ferricyanide technique). The dehydrogenases involved in the oxidation of glucose, lactate and pyruvate in presence of brain tissue are (relatively) narcotic-insensitive.

2. Anaerobic glycolysis by brain is not inhibited by relatively high concentrations of chloretone.

3. Anaerobic oxidations of glucose, lactate and pyruvate in the presence of brain tissue and of a carrier such as pyocyanine can be followed by the ferricyanide technique.

4. Anaerobic lactate oxidation by ferricyanide, in presence of crushed yeast, can be followed manometrically in the absence of added carriers. It is but feebly inhibited by relatively high concentrations of chloretone.

5. The addition of crushed yeast to skeletal muscle extract containing sodium α-glycerophosphate produces a narcotic-sensitive respiratory system.

6. The following systems which are but feebly narcotic-sensitive become highly narcotic-sensitive on addition to them of a cytochrome oxidase preparation, viz. dialysed skeletal muscle extract, cozymase and α-glycerophosphate; liver glucose dehydrogenase, cozymase and glucose.

These systems which can be studied manometrically under anaerobic conditions by the ferricyanide technique are not inhibited appreciably by concentrations of chloretone which exercise large inhibitions of the oxygen uptake when pig heart cytochrome oxidase is added.

7. Chloretone at relatively high concentrations does not inhibit the reduction of heart flavoprotein by reduced cozymase, or the reduction of methylene blue by reduced flavoprotein. It exercises a large inhibition of the oxygen uptake of a system composed of skeletal muscle extract, cozymase, flavoprotein, cytochrome oxidase and sodium lactate.

8. The evidence suggests that chloretone at biologically important concentrations affects largely either a special flavoprotein or some component of the tissue respiratory system which plays an intermediate role between flavoprotein and cytochrome oxidase.

Our thanks are due to the Medical Research Council for a grant in aid of the equipment of this laboratory, to the Society for the Protection of Science and Learning for a grant to one of us (M. M.), and to Dr P. J. G. Mann for his help in some of the experimental work described in this paper. We are also grateful to the Ella Sachs Plotz Foundation for a grant for the purchase of animals.

REFERENCES

Booth & Green (1938). *Biochem. J.* **32**, 855.
Corran, Green & Straub (1939). *Biochem. J.* **33**, 793.
Davies & Quastel (1932). *Biochem. J.* **26**, 1672.
Dewan & Green (1938). *Biochem. J.* **32**, 626.
Green, Needham & Dewan (1937). *Biochem. J.* **31**, 2327.
Haas (1937). *Biochem. Z.* **291**, 79.
—— Horecker & Hogness (1940). *J. biol. Chem.* **136**, 747.
Harrison (1931). *Biochem. J.* **25**, 1016.
Hawthorne & Harrison (1939). *Biochem. J.* **33**, 1573.
Jowett (1938). *J. Physiol.* **92**, 322.
—— & Quastel (1937). *Biochem. J.* **31**, 565.
Keilin & Hartree (1938). *Proc. roy. Soc.* B, **125**, 171.
—— —— (1939). *Proc. roy. Soc.* B, **127**, 167.
Lipmann (1939). *Nature, Lond.*, **143**, 436; *Enzymologia* (1937), **4**, 65.
McClure, Hartmann, Schnedorf & Schelling (1939). *Ann. Surg.* **110**, 836.
Quastel (1939). *Physiol. Rev.* **19**, 135.
—— & Wheatley (1932). *Proc. roy. Soc.* B, **112**, 60.
—— —— (1934). *Biochem. J.* **28**, 1521.
—— —— (1938). *Biochem. J.* **32**, 936.
Sen (1931). *Biochem. J.* **25**, 849.
Straub (1939). *Biochem. J.* **33**, 787.
Theorell (1936). *Biochem. Z.* **288**, 317.

54. PROTEIN CATABOLISM AND PROTEIN SPARING UNDER ADRENALINE

By CHARLES REID

From the Department of Physiology, London Hospital Medical College, University of London

(*Received 28 March 1941*)

It has been known for many years that a subcutaneous injection of adrenaline is followed by a rise in O_2 consumption [La Franca, 1909]. Views are not unanimous regarding the nature of the foodstuffs oxidized to furnish the additional calories. To Cori [1931] it appears that under the influence of adrenaline, although the oxidation of carbohydrate is increased a relative suppression of the oxidation of carbohydrate occurs in the intact animal, even in the presence of a carbohydrate plethora, with the result that more than half of the extra calories are supplied by the oxidation of fat [Cori & Cori, 1928]. It is clear, however, from recent work on the isolated heart by Cruickshank & Startup [1934] and by Bogue *et al.* [1935] that the heart under adrenaline uses more O_2 and that the increased O_2 is used preferentially for the combustion of glucose, provided that sufficient glucose is present in the perfusing fluid. Moreover, Paton [1903] and Underhill & Closson [1906] found an increased breakdown of protein under adrenaline when the calorie value of the diet was inadequate.

The fact that adrenaline increases the blood sugar whereas insulin decreases it shows that these hormones have opposing effects as judged by the behaviour of the blood sugar, but the different behaviour of the blood sugar under adrenaline and insulin does not justify the conclusion that the metabolic actions of adrenaline and insulin are necessarily antagonistic as far as the utilization of carbohydrate is concerned.

The administration of carbohydrate lowers protein catabolism in the normal organism. This can readily be shown by giving carbohydrate to a normal fasting animal when the excretion of N promptly decreases. On the other hand, the excretion of N is not lessened by giving carbohydrate to a fasting depancreatized animal. Evidently the protein-sparing action of carbohydrate is linked in some way with the physiological role of insulin. If then adrenaline increases the breakdown of protein in the normal organism but the concomitant administration of carbohydrate prevents this, either largely or entirely, obviously adrenaline does not prevent the protein-sparing action of carbohydrate and so cannot be said to act strictly as an 'anti-insulin' hormone.

METHODS

A. *Experiments on anaesthetized cats*

Cats were fasted for 48 hr. and, after anaesthetization with chloralose, were given an intraduodenal injection of about 20 ml. warm water to maintain the flow of urine. After 2 hr. the bladder was emptied and the urine collected for an initial period of 3 hr. Then one of the following was given by slow infusion into a vein during a period of 5–6 hr., different animals being used for each kind of infusion: (1) adrenaline; (2) glucose; (3) adrenaline + glucose; (4) adrenaline + glucose + insulin; (5) saline. Samples were taken at the beginning and at the end of the 5–6 hr. period for determinations of the blood sugar, liver glycogen, urine sugar and inorganic S. The rate of excretion of inorganic S under the above conditions was taken as indicating the rate of protein catabolism [Reid, 1936] in preference to that of the excretion of N, which is affected considerably by variations in the rate of urine flow. Observations were also made on previously depancreatized cats. The body temperature of the anaesthetized animals was kept constant by means of a rectal thermometer which acted as a thermoregulator for the animal table [Fuchs & Reid, 1937–8].

RESULTS

In intact fasting cats the excretion of inorganic S is increased by infusing adrenaline into a vein [Table 1 (1)].

The increase varies in general with the dose of adrenaline used, viz. 0·02–0·08 mg./kg. body weight/hr. [Table 1 (1)].

Table 1

Nature of infusion			Average % change in hourly rate of excretion of inorganic S during perfusion as compared with control period
Adrenaline mg./kg./hr.	Glucose g./kg./hr.	Insulin unit/kg./hr.	
(1) Intact cats: 6 experiments in each group			
Saline control	–	—	0
0·02		-	+22
0·05	—	—	+39
0·08	–	—	+77
0·05	0·30	—	+26
0·08	0·40	—	+32
—	0·26	—	−15
—	0·40	—	−22
0·02	0·40	0·32	−18
0·07	0·60	0·50	+ 8
(2) Previously depancreatized cats: 6 experiments in each group			
0·04	—	—	+53
0·04	0·40	—	+56
0·04	0·40	0·50	+ 2

Thus adrenaline increases the rate of protein catabolism in intact fasting cats.

The administration of glucose along with adrenaline lessens the expected rise in the output of inorganic S [Table 1 (1)].

Glucose, therefore, can spare protein in an animal under the influence of adrenaline.

In previously depancreatized cats adrenaline increases the output of inorganic S [Table 1 (2)] and therefore the rate of protein catabolism, but protein is spared by giving glucose + insulin though not by glucose alone.

It thus seems reasonable to conclude that adrenaline is not strictly an anti-insulin hormone because it does not suppress the ability of the normal organism to use carbohydrate to spare protein. This tentative conclusion is further tested in the following experiments on non-anaesthetized dogs.

B. *Experiments on non-anaesthetized dogs*

Two bitches were subjected during 2 years to the following regime every 2 weeks with or without the administration of adrenaline:

Ordinary feeding for 10 days adjusted so as to keep their weights nearly constant throughout.

Deprivation of food, but not of water, for 2 days.

Catheterization to empty the bladder, with a further 2 days for the collection of urine during this period, and daily feeding by stomach tube with water or glucose solution.

Analysis of the 48 hr. urines for total N, inorganic S and glucose.

When adrenaline was used, it was given subcutaneously in doses of 0·25–1·0 mg. every 3 hr. Auer & Meltzer [1916] have shown that the effects of a subcutaneous dose of adrenaline last for several hours owing to slow absorption. As judged by the behaviour of the pulse rate, body temperature etc. after subcutaneous adrenaline, this certainly appears to be the case. Tissue necrosis may occur with the larger doses used unless injected at more than one site.

Table 2

	Dog *B* (11–12 kg.)		Dog *C* (15–16 kg.)	
Nature of experiment	Total N g./day	Inorganic S g./day	Total N g./day	Inorganic S g./day
Control	2·74	0·104	3·63	0·126
Adrenaline, 0·5 mg./3 hr.	3·29	0·132	4·42	0·147
Adrenaline, 0·5 mg./3 hr. +50 g. glucose daily	2·51	0·097	3·66	0·135
Control	2·68	0·092	3·60	0·118
Glucose, 50 g. daily	2·01	0·071	3·13	0·100
Adrenaline, 0·75 mg./3 hr. +50 g. glucose daily	2·87	0·107	3·80	0·129
Glucose, 80 g. daily	1·90	0·065	2·61	0·077
Adrenaline, 0·75 mg./3 hr.	3·40	0·121	4·08	0·123
Control	2·50	0·080	3·43	0·108

Results

Table 2 gives the data obtained from one of the 4 anoestrous periods in each bitch. Results from periods complicated by oestrus have been excluded. It is clear that, when adrenaline is given by subcutaneous injection to fasting dogs, the urinary output of total N and inorganic S rises, indicating that under these conditions protein catabolism increases. When glucose is given by stomach tube while the animals are under the influence of adrenaline, the urinary output of total N and inorganic S is significantly lessened, so that clearly protein can be spared by carbohydrate even during the acceleration of metabolic processes by adrenaline. The urine was free from sugar except on two occasions when small losses of less than 3 g. were found. The normal fasting dog likewise can use carbohydrate to spare protein (Table 2). Therefore the protein-sparing action of carbohydrate, which appears to be linked with the physiological role of insulin, is not prevented by adrenaline. The latter, accordingly, cannot be regarded strictly as an 'anti-insulin' despite the opposing effects of adrenaline and insulin on the blood sugar.

SUMMARY

Adrenaline has been shown to increase the catabolism of protein, but protein can still be spared by giving carbohydrate, even when the metabolic rate has been raised by adrenaline. The protein-sparing action of carbohydrate appears to be linked with the physiological role of insulin, since it is not shown under ordinary conditions by the depancreatized animal without insulin. Since the protein-sparing action of carbohydrate still functions when adrenaline is given, adrenaline and insulin do not have strictly antagonistic physiological roles despite their opposing actions on the blood sugar.

REFERENCES

Arer & Meltzer (1916). *Proc. Soc. exp. Biol. N.Y.* **14**, 54.
Bogue, Evans, Grande & Hsu (1935). *Quart. J. exp. Physiol.* **25**, 213.
Cori (1931). *Physiol. Rev.* **11**, 143.
—— & Cori (1928). *J. biol. Chem.* **79**, 343.
Cruickshank & Startup (1934). *J. Physiol.* **80**, 179.
Fuchs & Reid (1937–8). *J. Physiol.* **91**, 10*P*.
La Franca (1909). *Z. exp. Path. Ther.* **6**, 1.
Paton (1903). *J. Physiol.* **29**, 286.
Reid (1936). *J. Physiol.* **87**, 113, 121.
Underhill & Closson (1906). *Amer. J. Physiol.* **17**, 42.

55. A SIMPLIFIED ESTIMATION OF LACTATE IN MUSCLE

By R. E. JOHNSON and A. N. BARGER

From the Fatigue Laboratory, Harvard University, Boston, Massachusetts, U.S.A.

(Received 10 March 1941)

Edwards [1938], modifying the method of Friedemann *et al.* [1927], improved the apparatus and simplified the method for estimating lactate in normal human blood. Treatment of blood extracts with copper sulphate and copper hydroxide, preliminary boiling or aeration of extracts and cleaning out the apparatus or adding fresh reagents between runs were all found to be unnecessary, since none of these procedures had any significant effect either on the estimation of preformed lactate or on the recovery of added lactate. We have applied these modifications to deproteinized extracts of muscles, and have also used a simple apparatus for preparing such extracts. The present method can be used with any apparatus suitable for the method of Friedemann *et al.* [1927].

Materials

These are prepared according to Friedemann *et al.* [1927] except as noted below. Instead of sodium bisulphite, a 5 % aqueous solution of sodium metabisulphite is used. This gives slightly better recoveries, especially in warm weather. Excess bisulphite in the titration is removed with $N/3$ iodine in KI. Technical grades of iodine and KI are satisfactory and cost about one-half as much as CP grades. Hydrated disodium phosphate (USP powdered) is used for the adjustment of alkalinity in estimating bound bisulphite. If the product as bought does not contain enough water of crystallization to lower the temperature of water as it dissolves, it is dissolved in an equal weight of hot water and allowed to crystallize with constant stirring. The crystals so obtained need not be pulverized.

A stock solution of trichloroacetic acid, made by dissolving one part of acid by weight with 1·5 parts of water, is stored in the ice box and diluted one to ten as needed. As Wendel [1931–32] pointed out, this reagent may have a high blank. Baker's CP product usually has a blank no higher than that of distilled water, although some samples have a small, but not troublesome, blank. Trichloroacetic acid can be purified by sublimation, but this is a wasteful procedure.

Method

The tissue grinder described by Potter & Elvehjem [1936] was found by Newman [1938] to be well suited for quantitative extraction of lactate from muscle. It consists of a pyrex test tube of 30 ml. capacity, graduated at 25 ml. and fitted with a revolving glass pestle that has a 15 cm. glass shaft on its upper end.[1] The shaft fits by means of a rubber stopper to the shaft of a 1/50 H.P. electric motor that has a built-in reducing gear of 8½ ratio and a suitable rheostat.

A tube is set in a pan of solid CO_2 and 2 ml. of 4 % trichloroacetic acid are frozen in the bottom. The muscle is frozen in powdered CO_2 and a piece weighing about 250 mg. is weighed, while frozen, with a rapid torsion balance. It is dropped into the tube, covered with 3 ml. of ice-cold 4 % trichloroacetic acid and the whole mass is frozen solid. The tube is dipped into cold water until melting just begins, the pestle is inserted, the motor started and the muscle is ground to a fine frozen pulp. This takes about 2 min. The pestle is withdrawn and washed off quantitatively into the tube with ice-cold 4 % trichloroacetic acid. The tube is filled to 25 ml. with the same reagent, a clean rubber stopper is inserted and the mixture is shaken. It is then centrifuged for 5 min. at about 2500 r.p.m.

---

[1] These may be bought from Macalaster-Bicknell Co., Cambridge, Mass., U.S.A.

A suitable aliquot of the supernatant fluid is taken for lactate estimation according to Edwards [1938], with certain precautions. The water leaving the condensers should range from 30° to 40°. Below 30° aldehyde is retained; above 40° aldehyde is lost. This has been pointed out by Friedemann & Kendall [1929] and by Friedemann & Graeser [1933]. They have also advised cooling the tube in which the titration takes place when the room temperature is above 26°. Otherwise blanks are high and recoveries of standard lactate low. It is for this reason that we use completely hydrated disodium phosphate which has a negative heat of solution [Lehnartz, 1928].

Edwards [1938] found that as many as eleven runs could be made without cleaning out the apparatus or adding fresh manganous sulphate and H_2SO_4 between runs. We have extended his findings on this point to muscle. Friedemann *et al.* [1927] stated that for relatively small amounts of lactate, such as are found in 200 mg. of muscle, satisfactory results can be obtained without the use of manganous sulphate, provided that the rate of oxidation is carefully adjusted.

In our battery of 10 units, each run contains a reagent blank, a standard of lithium lactate and 4 muscle extracts in duplicate. The recovery of the standard is always in the range 97–102 % of the theoretical amount. The blank is usually less than 0·04 ml. of 0·005*N* iodine, and rarely as much as 0·06 ml. Duplicates of the extracts always agree to within 0·02 ml. of 0·005*N* iodine. These blanks and recoveries compare favourably with Wendel's [1931–32], although our blanks are slightly higher than his. He got recoveries of only 95 % of the theoretical values when adding zinc lactate, which was thought to be impure. The sensitivity of the method in our hands is therefore about the same as in his, namely, down to about 0·01 mg. of lactic acid, corresponding to not more than 5 mg. of lactic acid per 100 g. of muscle, with duplicate estimations agreeing to within 2 mg. of lactic acid per 100 g.

By the present method, duplicate estimations on each of the 4 gastrocnemii of two frogs, along with a reagent blank and a standard solution, can be finished in from 70 to 90 min. after the animals are killed. With an accumulation of samples, one run is made while another is being titrated, so that 10 titrations can be finished every 30–40 min., and since cleaning out the apparatus, or adding fresh reagents, is unnecessary, there is no delay in beginning new runs.

Evidence justifying the various steps of the method

In methodological studies, it is necessary to have a supply of material so homogeneous that the concentration of the substance in question is constant from one sample to another. In the case of muscle, this is best achieved by powdering the muscle. We have used pills made from frozen frog muscle. In the course of preparation, all the equipment is cooled with solid CO_2. The muscles are frozen and powdered in a mortar and the powder is sifted first through a coarse and then a fine sifter. The pills are made in a pill press so constructed that they are hammered solid by a steel plug fitting into a hole bored through a block of steel. They weigh between 200 and 300 mg. Duplicate estimations are made on each pill. The pills can be stored for days in airtight containers surrounded by solid CO_2 without change in content of water, glycogen or lactate. The lactate concentration is satisfactorily constant from pill to pill, the maximum difference in any given batch being 5 mg./100 g.

In taking an aliquot from the supernatant fluid, the assumption is made that all of the lactate originally present in the muscle is distributed in equal concentration by volume throughout both the supernatant fluid and the mass of denatured protein. Experiment 1 (Table 1) shows that in the present method this assumption is valid. The experiments were carried out on pills of the same batch, divided into two groups. The first group of analyses was made on aliquots taken from the supernatant fluid. The pills of the second group were ground up and the supernatant fluid was saved. Then each pill residue was extracted with three 5 ml. portions of the trichloroacetic acid. The original supernatant fluid and the washings were pooled, made up to known volume, and aliquots taken for analysis. Data of Newman [1938] are also included. The same result was obtained by these two methods of lactate extraction.

The three reagents most commonly used for deproteinizing muscle are trichloroacetic acid, mercury salts and sodium tungstate. Trichloroacetic acid in concentrations varying from 4 to 10 % has been used by Cori & Cori [1932–33], Davenport & Davenport [1928], Flock *et al.* [1939], Gordon & Quastel [1939],

Table 1. *Various factors influencing the estimation of muscle lactate*

Results for solutions of lithium lactate are expressed as mg. lactic acid per 100 ml., and are the average of duplicate or triplicate estimations.

Results for muscle and muscle pills are expressed as mg. lactic acid per 100 g. tissue, and are the average of duplicate or triplicate estimations on each of two or three samples.

Type of sample and treatment		Lactate content mg./100 ml. or g.	Lactate added mg./100 ml. or g.	Lactate recovered	
				mg./100 ml. or g.	% of amount added
	Exp. 1				
Batch 3 of muscle pills	quantitative extraction	55	—	—	—
	aliquots of supernatant	55	—	—	—
Batch 4 of muscle pills	quantitative extraction	48	—	—	—
	aliquots of supernatant	49	—	—	—
Rat muscle 1	quantitative extraction	42	—	—	—
	aliquots of supernatant	38	—	—	—
Rat muscle 2	quantitative extraction	47	—	—	—
	aliquots of supernatant	43	—	—	—
Rat muscle 3	quantitative extraction	39	—	—	—
	aliquots of supernatant	40	—	—	—
	Exp. 2				
4 % trichloroacetic acid:					
No added lactate		49	—	—	—
Lactate added		76	28	27	96·5
Lactate added		102	52	53	102
Quantitative extraction with acid sodium tungstate, followed by treatment with copper-lime:					
No added lactate		48	—	—	—
Lactate added		96	48	48	100
Quantitative extraction with acid mercuric sulphate followed by treatment with copper-lime:					
No added lactate		47	—	—	—
Lactate added		99	53	52	98

Table 1 (*cont.*)

Type of sample and treatment	Lactate content mg./100 ml. or g.	Lactate added mg./100 ml. or g.	Lactate recovered	
			mg./100 ml. or g.	% of amount added
Exp. 3				
Solutions of pure lithium lactate:				
In distilled water	—	1·91	1·91	100
In 4 % trichloroacetic acid	—	1·91	1·92	100·5
In 8 % trichloroacetic acid	—	1·91	1·89	99
In 16 % trichloroacetic acid	—	1·91	1·89	99
In distilled water	—	0·48	0·48	100
In 4 % trichloroacetic acid	—	0·48	0·49	102
In 8 % trichloroacetic acid	—	0·48	0·48	100
Pills of batch 4 ground in trichloroacetic acid:				
Ground in 10 times by wt. of 40 %, then diluted to 1 : 100 by wt.	54	—	—	—
Ground in 1 : 100 by wt. of 2 %	54	—	—	—
Ground in 1 : 200 by wt. of 2 %	58	—	—	—
Ground in 20 times its wt. of 4 %	54	—	—	—
Ground in 40 times its wt. of 4 %	53	—	—	—
Ground in 100 times its wt. of 4 %	57	—	—	—
Ground in 200 times its wt. of 4 %	55	—	—	—
Ground in 100 times its wt. of 8 %	57	—	—	—
Extracted quantitatively with 4 successive washings with 4 % trichloroacetic acid	55	—	—	—
Pills of batch 1 ground with 100 times their wt. of 4 % trichloroacetic acid:				
No added lactate	113	—	—	—
Added lactate	321	214	208	97
Pills of batch 2 ground with 100 times their wt. of 4 % trichloroacetic acid				
No added lactate	139	—	—	—
Added lactate	247	106	108	101·5
Pills of batch 5 ground in 100 times their wt. of 4 % trichloroacetic acid:				
No added lactate	49	—	—	—
Added lactate	111	61	62	101·5
Added lactate	73	24	24	100
Exp. 4				
4 % trichloroacetic acid extract of frog muscle, analysed with potassium permanganate by present method:				
No added lactate	383	—	—	—
Added lactate	591	211	208	98·5
Same extract, analysed with ceric sulphate according to Gordon & Quastel [1939]:				
No added lactate	383	—	—	—
Added lactate	698	320	315	98·5

Long & Grant [1930] and Long & Horsfall [1932] and others. Mercuric salts have been used by Lehnartz [1928], Kerly & Ronzoni [1937], Ronzoni & Wallen-Lawrence [1927], Ronzoni *et al.* [1928] and Lundsgaard [1933]. Sodium tungstate has been used much more with blood than with muscle.

Exp. 2 demonstrates that muscle extract is adequately deproteinized by any one of the foregoing methods. Pills of the same batch were divided into three groups. The first were ground and extracted with trichloroacetic acid, as described above. The second were treated with 1 ml. of 10 % sodium tungstate, 1 ml. of $2/3N$ H_2SO_4 and 8 ml. of water. After centrifuging, the supernatant fluid was saved and the residues were extracted with three 5 ml. portions of the acid-tungstate mixture. The supernatant fluid and the washings were pooled, diluted to known volume and treated with copper sulphate and calcium hydroxide, after Van Slyke [1917]. The pills of the third group were extracted with four 5 ml. portions of 4 % mercuric sulphate in N H_2SO_4. The four extracts were pooled, treated with H_2S and centrifuged. The supernatant fluid was aerated until the H_2S was removed. Aliquots were made slightly alkaline with NaOH and treated with copper sulphate and calcium hydroxide. In each group, to certain pills lithium lactate in amounts comparable with the lactate already present was added. Exp. 2 shows that the estimated lactate was the same regardless of the type of protein precipitant, and also that recovery of added lactate was satisfactory. This has previously been shown for blood by Ronzoni & Wallen-Lawrence [1927], although they did not use trichloroacetic acid, by Edwards [1938] for blood and by Davenport & Davenport [1928] for muscle.

Table 2. *Comparison of results with and without preliminary aeration and treatment with copper-lime*

Results represent the averages of duplicate or triplicate estimations.

	No preliminary boiling and aeration		10 min. preliminary boiling and aeration	
Species	No Cu-lime mg./100 g.	Cu-lime mg./100 g.	No Cu-lime mg./100 g.	Cu-lime mg./100 g.
Frog	146	143	147	145
,,	41	39	40	39
,,	177	177	179	175
,,	13	11	11	13
Guinea-pig	99	—	—	103
Man	30	—	—	29
Rabbit	95	—	—	92
Rat	143	—	—	138

It is well known that trichloroacetic acid may be used in wide concentrations and amounts without affecting the recovery of added lactate so long as there is enough to precipitate all the protein [Cori & Cori, 1932–33; Avery *et al.* 1935]. Exp. 3 (Table 1), containing data obtained from solutions of pure lithium lactate and from muscle, further demonstrates that over a range of 4 to 16 % concentration of the reagent, lactate may be accurately estimated and recovery of added lactate is satisfactory. It should be emphasized that it is necessary to have trichloroacetic acid with a small blank before such consistently satisfactory results are obtained. We have fixed on 4 % trichloroacetic acid only because of its convenience, and have made up 250 mg. of muscle to a volume of 25 ml. only to avoid the error inherent in pipetting small volumes.

The evidence that with muscle, as with blood, preliminary boiling and aeration and treatment with copper sulphate and calcium hydroxide are unnecessary is obtained in Table 2. Extracts of several frog muscles were prepared with 4 % trichloroacetic acid along with suitable reagent blanks. One-half of each extract was treated with 10 % copper sulphate and 20 % calcium hydroxide. The proportions were 25 volumes of extract, with enough 40 % NaOH to make it neutral, 10 volumes of copper sulphate, 10 volumes of calcium hydroxide and water to make 50 volumes. The mixture was allowed to stand at least one-half hour, with shaking every 5 min. It was centrifuged and aliquots of the supernatant fluid taken for analysis. An analysis of each type of extract was made with and without the preliminary boiling and aeration that are customary. The same results were obtained with and without treatment with copper and lime and with and without preliminary boiling and aeration. The table includes also data derived from the muscles of several other animal species. These data do not include estimation of the untreated extract with preliminary boiling, or estimation of the treated extract without preliminary boiling, since the results led to the same conclusion as those from frog muscle. The suggestion that treatment with copper and lime may be dispensed with under certain circumstances has been made by Friedemann *et al.* [1927], Friedemann [1928], Friedemann & Kendall [1929], Boyland [1928], Scott & Berg [1936] and Edwards [1938]. One of the advantages claimed for methods such as those used by Gordon & Quastel [1939], Lehmann [1938], Miller & Muntz [1938] and Rappaport & Reifer [1937], which differ in principle from that of Friedemann *et al.* [1927], is that removal of carbohydrates before analysis is unnecessary. We consider that with muscle it is not necessary even for the method of Friedemann *et al.* [1927].

We have tried repeatedly without success to detect in trichloroacetic acid extracts of frog muscles any appreciable amount of preformed volatile substances that will bind bisulphite.

Gordon & Quastel [1939] introduced the use of ceric sulphate instead of potassium permanganate as oxidant, claiming the advantage that treatment of muscle extracts with copper sulphate and calcium hydroxide is thereby rendered unnecessary. Exp. 4 compares their method with ours for the same muscle extract. The present method requires one-fourth of the time, employs simpler apparatus and gives the same results as that of Gordon & Quastel [1939].

SUMMARY

1. The simplifications which Edwards [1938] introduced into the method and apparatus of Friedemann *et al.* [1927] for estimating lactate in normal human blood are applicable also to muscle. Treatment of muscle extracts with copper sulphate and calcium hydroxide, as well as preliminary boiling and aeration, may be dispensed with. From five to ten estimations can be made without cleaning out the apparatus or adding fresh reagents.

2. A rapid, accurate method for preparing protein-free extracts of muscle, which uses the tissue grinder of Potter & Elvehjem [1936], is described.

3. No difference in the estimated preformed lactate or in recovery of added lactate is found when either trichloroacetic acid, mercuric sulphate or acid sodium tungstate is used for deproteinizing.

4. The use of ceric sulphate as oxidant is no more precise or convenient than the use of potassium permanganate and requires more time and more complicated apparatus.

REFERENCES

Avery, Kerr & Ghantus (1935). *J. biol. Chem.* **110**, 637.
Boyland (1928). *Biochem. J.* **22**, 236.
Cori & Cori (1932–33). *J. biol. Chem.* **99**, 493.
Davenport & Davenport (1928). *J. biol. Chem.* **76**, 651.
Edwards (1938). *J. biol. Chem.* **125**, 571.
Flock, Ingle & Bollmann (1939). *J. biol. Chem.* **129**, 99.
Friedemann (1928). *J. biol. Chem.* **76**, 75.
—— Cotonio & Shaffer (1927). *J. biol. Chem.* **73**, 335.
—— & Graeser (1933). *J. biol. Chem.* **100**, 291.
—— & Kendall (1929). *J. biol. Chem.* **82**, 23.

Gordon & Quastel (1939). *Biochem. J.* **33**, 1332.
Kerly & Ronzoni (1937). *J. biol. Chem.* **103**, 161.
Lehmann (1938). *Skand. Arch. Physiol.* **80**, 237.
Lehnartz (1928). *Hoppe-Seyl. Z.* **179**, 1.
Long & Grant (1930). *J. biol. Chem.* **89**, 553.
—— & Horsfall (1932). *J. biol. Chem.* **95**, 715.
Lundsgaard (1933). *Biochem. Z.* **264**, 209.
Miller & Muntz (1938). *J. biol. Chem.* **126**, 413.
Newman (1938). *Amer. J. Physiol.* **122**, 359.
Potter & Elvehjem (1936). *J. biol. Chem.* **114**, 495.
Rappaport & Reifer (1937). *Microchim. Acta*, **2**, 62.
Ronzoni, Glaser & Barr (1928). *J. biol. Chem.* **80**, 309.
—— & Wallen-Lawrence (1927). *J. biol. Chem.* **74**, 363.
Scott & Berg (1936). *J. biol. Chem.* **115**, 163.
Van Slyke (1917). *J. biol. Chem.* **32**, 455.
Wendel (1931–32). *J. biol. Chem.* **94**, 717.

56. THE MICRO-DETERMINATION OF POTASSIUM

By C. R. HARINGTON

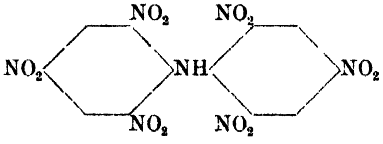
From the Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

(*Received 26 March 1941*)

The determination of potassium is more troublesome than that of any other biologically important cation; at the same time there are many conditions involving variations in the electrolyte content of the body fluids in which accurate estimations of potassium are desirable. It therefore appeared worth while to try to devise an analytical method for potassium which should be simpler than those at present in use; this paper describes a new method which offers certain advantages of simplicity whilst giving a degree of accuracy not significantly less than those claimed for the more time-consuming procedures.

The methods at present available for the determination of potassium fall into two groups: (1) determination as the chloroplatinate and (2) determination by one of the various modifications of the cobaltinitrite procedure [compare Peters & Van Slyke, 1932]. The chloroplatinate method is excellent for inorganic analysis when reasonably large quantities of material are available; it loses accuracy however when adapted to micro-analysis. The cobaltinitrite methods which have been most widely employed in biological work suffer from the theoretical disadvantage that there is uncertainty in the composition of the final precipitate; this means that although a series of analyses conducted by one worker under constant conditions may give mutually consistent results with a small margin of error, the basis of the method is not satisfactory.

In the search for a new procedure attention was directed to the possible use of dipicrylamine; this compound, di(2:4:6-trinitrophenyl)amine, forms a deep



orange-red potassium salt, of which the solubility in water is similar to that of potassium sodium cobaltinitrite; it has been proposed as a qualitative reagent for the detection of small amounts of potassium [Feigl, 1937] and is stated to be specific for potassium in presence of other metals of the 4th and 5th groups except rubidium and caesium and in absence of ammonium salts; no record has been found of its application to the quantitative determination of potassium.[1]

Preliminary experiments showed that potassium dipicrylamine was soluble in alcohol, to give, in low concentrations, solutions of a strong orange colour; this suggested the possibility of a determination of potassium based on measurement of the colour of an alcoholic solution of the precipitate of potassium dipicrylamine obtained from the solution to be analysed.

---

[1] Since this was written my attention has been called to two papers on the determination of potassium with the aid of dipicrylamine by Kolthoff & Bendix [1929] and Amdur [1940]; these papers do not however describe the application of the method to the analysis of blood.

EXPERIMENTAL

Colorimetric determination of potassium dipicrylamine

It was first necessary to ascertain the possibility of accurate measurement of the colour. For this purpose a series of standard solutions of potassium dipicrylamine in alcohol was prepared as follows. Commercial dipicrylamine was recrystallized from acetic acid; a solution of the sodium salt was made by dissolving the dipicrylamine (0·44 g.) in boiling $N/10$ sodium carbonate (20 ml.) and filtering the cooled solution; a slight excess of this solution was added to one of pure potassium nitrate containing 1 mg. K/ml.; after keeping overnight in the ice chest the potassium dipicrylamine was collected, washed with ice water and recrystallized from boiling $N/100$ potassium carbonate; the recrystallized material was dried *in vacuo* over sodium hydroxide. A stock solution of the salt in alcohol was made which contained 0·02 mg. K/ml. and from this a series of dilutions was prepared.

The colours of these solutions were measured in two ways: (*a*) with the Zeiss step-photometer; (*b*) with a simple photoelectric device [compare Harington *et al.* 1940]. Using the step-photometer, the best results were obtained with filter S 53, having maximum transmission at about 530 mμ; with the photoelectric device the most favourable conditions so far found have been with a combination of Wratten filter no. 61 and 2 mm. thickness of Chance's calorex glass to absorb the infra-red to which the cell is sensitive.

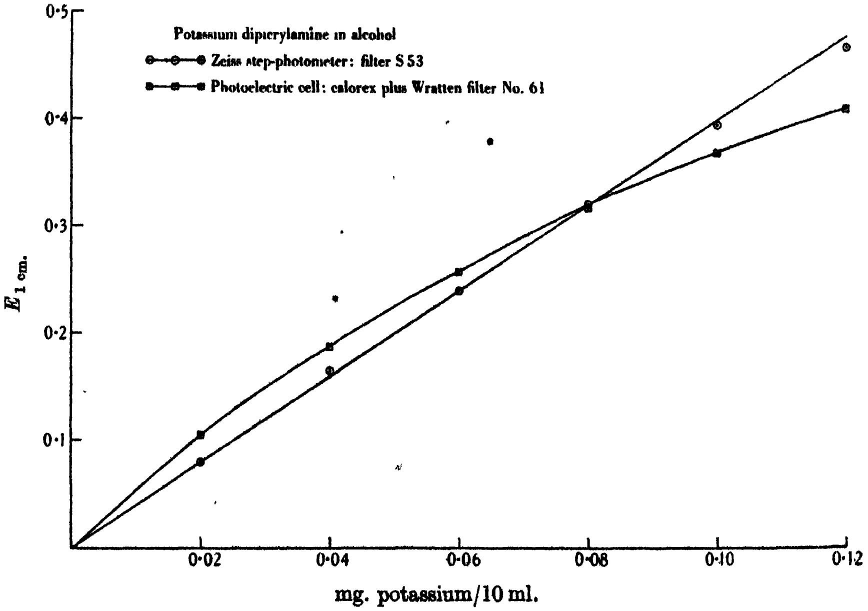


Fig. 1.

Results obtained by both these methods are shown in Fig. 1. It will be seen that over the range 0–0·1 mg. K/10 ml. the observations with the step-photometer are closely in accordance with Beer's law; at higher concentrations deviation from linearity became considerable. With the photoelectric device, on the other hand, a linear relationship between extinction coefficient and concentration of potassium is not obtained over any range; this is doubtless to be explained by incomplete elimination of the infra-red and by the breadth of the transmission band of the Wratten filter employed.

It is clear that if the colour is to be measured photoelectrically it will be necessary to work from a calibration curve such as that given in Fig. 1; with the step-photometer on the other hand, provided that the solution is adjusted to give a value of $E_{1\,\text{cm.}} < 0{\cdot}4$, the concentration of potassium is given by the formula

$$c = 2{\cdot}5E$$

where c is the concentration of potassium in mg./100 ml. in the final solution and E is the observed value of the extinction coefficient using filter S 53 and a 1 cm. cell.

The orange colour of the alcoholic solution of potassium dipicrylamine is not suitable for simple comparative colorimetry; such colorimetry can however be improved with the aid of suitable filters and fair results have been obtained with a Leitz Duboscq type of instrument using Wratten filter no. 61.

In all the analyses recorded below the colour measurements have been made with the step-photometer.

Analysis of solutions containing known amounts of potassium

The general method of analysis is as follows. The solution containing potassium at a concentration of 0·1–0·25 mg./ml. and at approximately neutral reaction is mixed with excess of the precipitating reagent (sodium or lithium dipicrylamine: see below); when crystallization of the potassium salt is complete it is collected, washed and dissolved in alcohol, the volume of the final solution being adjusted so that the value $E_{1\,\text{cm.}}$ observed in the step-photometer falls in the range 0·20–0·40; the colour is then measured.

(*a*) *Precipitating reagent.* In the earlier experiments this consisted of a solution of sodium dipicrylamine prepared as described above; later, for reasons which will appear, this was replaced by a similar solution of lithium dipicrylamine.

(*b*) *Time of precipitation.* When the precipitant is mixed with a solution containing potassium at about 0·1 mg./ml. concentration, crystallization of the potassium salt is best initiated by scratching the walls of the tube with a fine glass rod; the crystallization is completed in the ice chest. An experiment was performed to determine the time necessary for complete precipitation; the results are shown in Table 1, from which it appears that 2 hr. suffice; this period was therefore adopted as a routine.

Table 1. *Effect of time of precipitation on recovery of potassium*

Sample	Time in ice chest	K present mg.	K found mg.
A	1 hr.	0·200	0·188
B	2 hr.	0·200	0·198
C	3 hr.	0·200	0·193
D	Overnight	0·200	0·198

(*c*) ***Analysis of known solutions of potassium nitrate.*** A stock solution of pure potassium nitrate was prepared, containing 2 mg. K/ml.; from this a number of dilutions were made by an independent observer. The results obtained are shown in Table 2.

Table 2. *Analyses of solutions of potassium nitrate*

Sample	K present mg./100 ml.	K found mg./100 ml.	Error %
A	20·0	19·4	-3·0
B	21·6	22·4	+3·6
C	26·8	27·6	+4·3
D	38·0	36·3	-4·5
E	42·8	40·8	-4·7

From this it appears that the error in the determination on simple potassium nitrate solutions is within ±5 %.

(*d*) *Effect of other blood cations.* In order to study this point a series of solutions was analysed which all contained 20 mg. K/100 ml., but which differed in the amounts of Na, Ca and Mg which were present; the results are given in Table 3.

Table 3. *Effects of other blood cations on recovery of potassium*

Solution	Present mg./100 ml.				K found mg./100 ml.
	Na	K	Ca	Mg	
A	350	20	10	3	19·3
B	700	20	20	6	23·1
C	700	20	—	—	23·6
D	—	20	20	—	19·3
E	—	20	—	6	19·3

The recovery from solution A, which contains the amounts of the various cations found in normal blood serum is satisfactory; solution B on the other hand, in which all cations save K are doubled in concentration, gives a high result; a similarly high result is obtained with solution C containing no Ca or Mg but double the normal amount of Na, whilst correct results are obtained in presence of double the normal amounts of Ca and Mg when Na is absent (solutions D and E). It is clear that the greatest danger of interference comes from Na, and extension of the above experiment showed that such danger exists at levels of Na only slightly above the normal.

The above analyses were carried out with the sodium dipicrylamine precipitant in which the sodium ion concentration is $N/10$; it was evident that the danger of interference from sodium could be eliminated if a more soluble salt of dipicrylamine could be found; the lithium salt is in fact much more soluble than the sodium salt and a precipitating reagent was therefore prepared by dissolving 0·44 g. of dipicrylamine in 20 ml. of $N/10$ lithium carbonate; with the aid of this reagent it is possible to determine potassium accurately in presence of proportions of sodium greatly in excess of those found in biological fluids (Table 4).

Table 4. *Lithium dipicrylamine as precipitant; determination of potassium in presence of increasing amounts of sodium*

Solution	Na mg./100 ml.	K mg./100 ml.	K found mg./100 ml.
A	—	20·0	19·7
B	350	20·0	20·0
C	450	20·0	20·3
D	550	20·0	19·7
E	650	20·0	20·0

In view of these results the lithium dipicrylamine solution was adopted as precipitant in all future experiments.

Determination of potassium in blood serum

The analysis cannot be carried out directly on blood serum since the precipitation of potassium dipicrylamine is apparently inhibited by the organic constituents; the method adopted in the present work has been to incinerate a trichloroacetic acid filtrate of serum with sulphuric and nitric acids and to analyse the solution of the sulphate ash. This procedure has the advantages over dry incineration of greater rapidity and freedom from danger of loss of alkali chloride.

Reagents.

1. *Trichloroacetic acid*, 20 % aqueous solution.
2. *Sulphuric acid*, Analar, 4*N* solution.
3. *Nitric acid*, Analar, concentrated.
4. *Lithium dipicrylamine.* Commercial dipicrylamine is recrystallized from glacial acetic acid, 0·44 g. is dissolved by boiling in 20 ml. of *N*/10 lithium carbonate; the cooled solution is filtered.
5. *Potassium dipicrylamine*, saturated aqueous solution. The recrystallized salt is dissolved in warm *N*/1000 potassium carbonate in the proportion of 1 mg./ml. and the solution is kept in the ice chest until crystallization is complete; the mother liquor is used as washing fluid (see below).

Procedure.

In drawing the blood precautions must be taken to avoid haemolysis. 1 ml. serum is mixed with 3 ml. distilled water followed by 1 ml. 20 % trichloroacetic acid and the mixture is filtered. Of the filtrate 2·5 ml. (equivalent to 0·5 ml. serum) are placed in a 6 × 1 in. pyrex test tube together with 0·6 ml. 4*N* H_2SO_4 and a glass bead; the water is boiled off and the charred residue treated, after partial cooling, with one drop of concentrated HNO_3; heating is then resumed until the residue is colourless; the whole process is complete in a few minutes. The residue is then transferred quantitatively with small amounts of water to a platinum dish and evaporated as far as possible on the steam bath; excess of sulphuric acid is removed by cautious heating over the free flame, the contents of the dish being finally briefly but strongly ignited.

The ash is transferred with small quantities of water to a 3 × ⅜ in. test tube and the solution evaporated to 0·5–1·0 ml.; 1 ml. of the lithium dipicrylamine solution is added and crystallization of the potassium salt is initiated by scratching the tube with a fine glass rod. After 2 hr. in the ice chest the precipitate is collected on a small sintered glass funnel (1·0 cm. disk; porosity G 3 or G 4), the precipitation tube being carefully drained, and the precipitation tube and funnel are washed with two 1 ml. portions of the ice-cold saturated aqueous potassium dipicrylamine and finally with 1 ml. of ice-cold distilled water.

The receiver is then changed and the precipitation tube and funnel are washed through with warm alcohol, which dissolves the potassium salt; the alcoholic filtrate is made up to 15 ml. and the colour measured in the step-photometer. With normal samples of serum this will give a value of $E_{1\,\text{cm.}}$ between 0·24 and 0·32.

The results of a series of determinations on the sera of normal individuals are given in Table 5; the range observed, 17·5–24 mg./100 ml., is in accordance with the accepted values for normal serum potassium.

Table 5. *Potassium content of normal blood serum*

Sample no.	K found mg./100 ml.	Sample no.	K found mg./100 ml.
1	20·7	8	19·2
2	18·1	9	17·5
3	18·5	10	18·5
4	18·1	11	20·0
5	18·8	12	24·0
6	19·0	13	22·5
7	18·8	14	21·0

Recovery of potassium added to serum

In Table 6 are recorded the results of experiments in which analyses were made of a sample of serum before and after addition of measured amounts of potassium.

Table 6. *Recovery of potassium added to serum*

Sample no.	K added mg./100 ml.	K found mg./100 ml.	K recovered mg./100 ml.	Error %
1	0	20·7	—	—
2	10	31·3	10·6	+6·0
3	20	42·0	21·3	+6·5
4	40	59·5	38·8	−3·0

It will be seen that recoveries are satisfactory.

SUMMARY

A method for the determination of potassium is described which is based on precipitation as potassium dipicrylamine; the precipitate is collected, washed and dissolved in alcohol, and the potassium content determined by measurement of the colour of the resulting solution.

The application of the method to determination of blood serum potassium is described.

REFERENCES

Amdur (1940]. *Industr. engng. Chem.* Anal. Ed. **12**, 731.

Feigl (1937). Spot Tests, p. 144. New York: Nordemann Publishing Company.

Harington, Pochin & Squire (1940). *Clin. Sci.* **4**, 311.

Kolthoff & Bendix (1929). *Industr. engng. Chem.* Anal. Ed. **11**, 94.

Peters & Van Slyke (1932). Quantitative Clinical Chemistry, **2**, 741–55. London: Baillière, Tindall & Cox.

57. ESTIMATION OF VITAMIN A AND CAROTENE IN HUMAN BLOOD

By SIMON YUDKIN

From the Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

(*Received 31 March 1941*)

During the past few years numerous investigations have been carried out on the vitamin A and carotene contents of human blood in health and various diseases. Unfortunately, the method of estimation varies from author to author and in many cases the results are given in other than international units [Dost, 1937; Kimble, 1939; May *et al.* 1940], so that comparison is impossible. The following investigation was undertaken with a view to discovering the relative efficacies of the various methods of estimation. A preliminary series of normal blood values is also given.

Discussion of methods

The extraction is usually done on serum or plasma. Many authors saponify the serum with alcoholic KOH, some in the cold to eliminate the effect of haemolysis [Willstaedt & With, 1938], others on a boiling water bath for longer [Lindquist, 1938] or shorter [Pett & Le Page, 1940] periods. Clausen & McCoord [1938] and Kimble [1939] extract directly from serum precipitated with alcohol. The extraction is done either with ether or light petroleum and the resulting extract evaporated under nitrogen or carbon dioxide or in air.

The estimation of the vitamin A is usually carried out by means of the blue reaction of Carr and Price, but the absorption at 328 mμ is sometimes estimated directly [Chevallier & Choron, 1935]. The depths of the yellow colour of the carotene and the blue colour of the vitamin A reaction have been estimated in the following ways.

1. *Comparison in colorimeter with standard solutions* (potassium dichromate for carotene and either copper sulphate or a mixture of copper sulphate and cobalt nitrate [Schneider & Widmann, 1935] for the blue colour). The main criticism of this method is the fact that the colours of standard and unknown are not spectrographically the same. (Indeed 10 % copper sulphate solution may be used as part of the filter system in photoelectric colorimetry of the blue colour.) Besides this the rapid fading of the blue colour makes matching difficult.
2. *Lovibond tintometry.* The same criticisms apply (see Notevarp & Weedon [1936] for more detailed discussion of these points).
3. *The Zeiss step-photometer* is more accurate and fairly satisfactory but again colour matching is difficult because of fading.
4. *Spectrophotometry* at 328 mμ is accurate but needs complicated apparatus and there are several interfering factors at this wave-length.
5. *Photoelectric colorimetry.* This method is at present the most satisfactory. It eliminates the personal factor of colour matching and makes it easy to estimate the density of the blue colour at its real maximum. It should, however, be remembered that the solutions do not necessarily follow Beer's Law [Koehn & Sherman, 1940], so that a calibration curve or table must be constructed. The

best method of estimation would be photoelectric colorimetry with monochromatic light (a 'monochromator') but there is, as far as I know, no such instrument available.

For the present investigation a simple photoelectric colorimeter was therefore used, with combinations of filters with maximum transmission at 440 mμ for the carotene and 620 mμ for the blue colour of the Carr-Price reaction (Fig. 1).

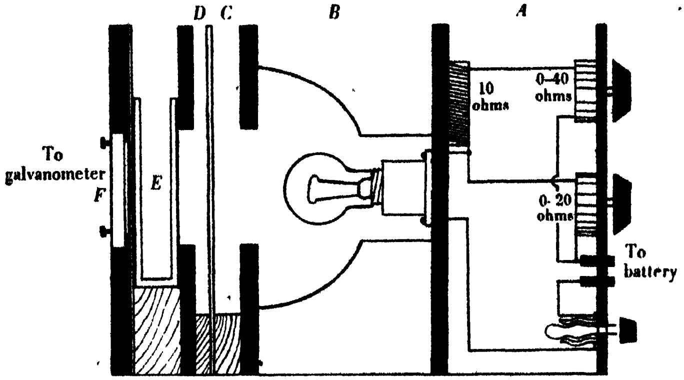


Fig. 1. Simple photoelectric colorimeter.

A. Simple circuit to give coarse and fine adjustment of illumination.

B. Source of illumination. 6 V. 3 W. Mazda lamp with small U-filament, run off four 2 V. cells to give increased proportion of blue light for the 440 mμ filter. Ordinary hemispherical torch reflector behind.

C. Space for filters. Filter 620 mμ maximum; Wratten 26 red gelatin filter combined with 1 mm. thickness of 'calorex' glass and 1 mm. thickness of 'signal green' glass, manufactured by Messrs Chance. Filter 440 mμ maximum; Wratten gelatin filters 35 and 2 A combined with 2 mm. thickness of 'signal green' glass.

D. Space for card to cut off all light falling on photocell when not in use. Thin glass plate between this and filters.

E. Glass cell holding test solutions. Volume about 4 ml. depth 10 mm. optically ground. Marked on edge at 0·3 ml. and 3·3 ml.

F. Photocell. Selenium barrier cell manufactured by Mr Wender. Size 1 cm. × 2 cm. Thin glass plate in front.

Galvanometer; mirror type; sensitivity 12 cm. deflexion at 1 m. per microamp. (Messrs Pye Ltd.).

A smaller glass cell and smaller photoelectric cell may be used. The volumes used in these experiments are the most suitable for the sizes given.

Calibration

Carotene. A solution of carotene in arachis oil[1] containing 200 international units (120 μg.) per g. was used. A weighed quantity was dissolved in light petroleum and aliquots made up to the 3·3 ml. mark in the standard cell. The deflexions on the galvanometer from the 'centre position' were noted and the calibration table constructed (Table 1).

Vitamin A. A solution was made of about 10 mg., accurately weighed, of pure vitamin A 2-naphthoate[1] in 25 ml. pure arachis oil containing a trace of quinol. 5 ml. of this solution were made up to 50 ml. with pure dried chloroform and aliquots of this were used for the Carr-Price reaction. (The actual volumes were aliquots made up to 0·3 ml. and 3 ml. of reagent added, but these depend on the size of the cell in the colorimeter) (Table 2).

Correction for blue colour produced by carotene. A solution of a weighed quantity of carotene was made in chloroform. Aliquots were made up to 0·3 ml.

---

[1] Kindly supplied by British Drug Houses Ltd., London.

and 3 ml. of antimony trichloride reagent were added in the colorimeter with the 620 mμ filter in place. The readings were noted and the calibration table constructed (Table 3).

Table 1. *Calibration table for pure β-carotene*

Filter 440 mμ

Vol. of aliquot in ml.	Galvanometer reading mm.	Log I_0/I	Total μg. in solution	Log I_0/I per μg.
0·05	92·5	0·0339	0·70	0·0487
0·1	85·5	0·0680	1·40	0·0485
0·2	74	0·1308	2·79	0·0468
0·3	64·5	0·1904	4·17	0·0454
0·4	57·5	0·2403	5·58	0·0431
0·5	51	0·2924	6·98	0·0419
0·6	45	0·3468	8·37	0·0414
0·7	41	0·3872	9·77	0·0397
0·8	38	0·4202	11·16	0·0377
0·9	35·5	0·4498	12·56	0·0367
1·0	33	0·4815	13·95	0·0345

Table 2. *Calibration table for vitamin A 2-naphthoate*

Filter 620 mμ

Vol. of aliquot in ml.	Galvanometer reading mm.	Log I_0/I	Total international units in solution	Log I_0/I per international unit
0·025	87	0·0648	2·20	0·0295
0·05	75·5	0·1264	4·40	0·0292
0·075	65	0·1914	6·61	0·0290
0·10	56	0·2561	8·81	0·0290
0·125	49	0·3141	11·01	0·0285
0·15	44	0·3608	13·21	0·0274
0·2	35·5	0·4553	17·62	0·0260
0·25	29·5	0·5360	22·02	0·0244
0·3	24·5	0·6151	26·43	0·0234

Vitamin A 2-naphthoate contains 2,225,000 international units vitamin A per g. [Mead *et al.* 1939].

Table 3. *Correction curve for blue colour produced by β-carotene*

Filter 620 mμ

Vol. of aliquot in ml.	Galvanometer reading mm.	Log I_0/I	Total μg. in solution	Log I_0/I per μg.
0·025	98	0·0079	0·92	0·00873
0·05	96·25	0·0168	1·84	0·00920
0·075	94·5	0·0246	2·76	0·00894
0·10	93·5	0·0339	3·68	0·00920
0·125	91·0	0·0410	4·60	0·00893
0·15	89	0·0504	5·52	0·00913
0·20	86	0·0655	7·36	0·00892
0·25	82·5	0·0835	9·20	0·00908
0·30	79·5	0·0996	11·04	0·00903

Comparison of methods for extraction from blood

Multiple samples of serum or plasma were extracted by each of the following four methods:

1. Preliminary saponification with alcoholic KOH on a boiling water bath followed by extraction with ether ('alkali digestion method') [De Haas & Meulemans, 1938; Lanzing, 1938; Van Eekelen & Emmerie, 1934]. (For details see Lindquist [1938].)

2. Preliminary saponification with alcoholic KOH on a boiling water bath followed by extraction with light petroleum [Pett & Le Page, 1940].

3. Extraction three times with ether after precipitation with alcohol; no saponification.

4. Extraction by light petroleum, after precipitation with alcohol; no saponification [Kimble, 1939; May *et al.* 1940; Clausen & McCoord, 1938]. (For details see later.)

The results are given in Table 4.

Table 4

Extraction of serum or plasma by different methods; results in μg. per 100 ml. for carotene and international units per 100 ml. for vitamin A.

		Saponification followed by				Simple extraction with			
		Extraction with ether		Extraction with light petroleum		Ether after precipitation with alcohol		Light petroleu after precipitati with alcohol	
Subject	Date	Carotene	Vitamin A	Carotene	Vitamin A	Carotene	Vitamin A	Carotene	Vitami
V. K. C.	9. i. 41	149	99	108	78	149	102	153	102
A. C.	11. i. 41	135	115	71	50	135	115	135	139
S. L.	22. i. 41	102	157	73	144	102	157	102	157
I. L.	3. ii. 41	118	180	76	132	118	152	116	169
J. D.	11. ii. 41	154	129	140	121	140	154	154	155
M. S.	14. ii. 41	151	150	106	152	113	161	140	161
J. S.	21. ii. 41	116	150	65	130	140	145	121	134
A. B.	24. ii. 41	124	140	40	84	94	140	134	138
B. C.	5. iii. 41	104	125	67	98	134	122	134	130
B. W.	5. iii. 41	50	96	27	90	50	118	66	128
C. D.	21. iii. 41	50	96	34	50	50	115	59	101
Pooled serum	10. ii. 41	170	381	76	234	170	370	160	375

It can be seen from Table 4 that saponification followed by extraction with light petroleum gives consistently lower results than any other method. (Incidentally I could not confirm that saponification for a longer time than 5 min. (see Pett & Le Page [1940]) had any effect on the final result.) Simple extraction either with ether or petroleum after precipitation gives results as good as or better than those obtainable by Lindquist's more complicated method. As the simple light petroleum extraction is the easiest of all it is used for the estimation in normal sera.

Procedure for estimation in blood

3 ml. of serum or plasma are put into a narrow-necked tube. 3 ml. of 95 % alcohol are added slowly with shaking, followed by 6 ml. of pure light petroleum (B.P. 40–60°) and the tube tightly corked. It is then shaken by hand for 10 min. The tube is centrifuged for 1 min. at low speed to separate the layers. The light petroleum layer is removed into another tube and a 5 ml. aliquot taken. This is evaporated in a gentle stream of carbon dioxide or nitrogen in a water bath at 30–40° and then at 70° for a few seconds. The residue is taken up in light petroleum and made up to 3·3 ml. in the standard cell. The deflexion of the galvanometer from the 'centre position' with the 440 mμ filter in place is noted and the result expressed in μg. of carotene per 100 ml. of serum. The solution is evaporated to dryness under carbon dioxide or nitrogen and the residue dissolved in chloroform and made up to the 0·3 ml. mark. The 620 mμ filter is put in and the light altered to the 'centre position'; the cell is put in and 3 ml. of antimony trichloride reagent added all at once. The reading at the point of temporary stability is noted and after correction for the amount of blue colour produced

by the carotene, the vitamin A is expressed in international units per 100 ml. of serum.

Notes. 1. The 'centre position' is found as follows. The cell containing pure petroleum up to 3·3 ml. is put in with the 440 mμ filter. The light is altered to give a reading of 100. The reading when the cell is removed is the 'centre position' for the 440 mμ filter. Similarly 0·3 ml. of chloroform and 3 ml. of antimony trichloride reagent are used with the 620 mμ filter.

2. The alcohol must be added slowly and the mixture shaken while adding. Also vigorous shaking by hand must be continued for at least 10 min. Estimations of the extract after different periods of shaking show that with the alcohol added slowly almost complete extraction occurs after 5 min., but when it is added all at once a tough precipitate forms which does not yield the maximum readings until shaking for 20 min. has been carried out. Mechanical shaking on a machine also gives low readings. Evaporation of the extract with air destroys some of the vitamin A but leaves the carotene unharmed.

3. The yellow pigment measured in serum extracts is not all carotene. The proportion of the different pigments, however, is not easy to discover and it varies from serum to serum. The correction for carotene is applied on the assumption that all the yellow colour is due to carotene. Even if only half is carotene the difference would usually be only about 10 or 15 units of vitamin A.

To check the efficiency of the method several further tests were made.

1. The residue after removal of the light petroleum was re-extracted with a further 6 ml. of light petroleum. The results were never higher than 5 μg. of carotene and 6 units of vitamin A per 100 ml. serum.

2. Duplicate samples never gave a difference of more than 2 in the galvanometer readings. This corresponds to an error of about ±3 %.

3. Extractions of different volumes of the same serum gave the same final results.

4. Slight degrees of haemolysis had no effect on the results.

5. Storage of the serum for times up to 48 hr. in a refrigerator at 2° had no effect and the results were the same as the estimations on fresh serum.

Table 5. *Normal values*

Subject	Date	Carotene in μg. per 100 ml. serum or plasma	Vitamin A in international units per 100 ml. serum or plasma
1	12. vii. 40	151	132
2	22. vii. 40	150	112
3	27. vii. 40	220	157
4	27. vii. 40	182	136
5	27. vii. 40	241	122
6	29. vii. 40	158	135
7	29. vii. 40	161	100
8	15. viii. 40	117	112
9	12. viii. 40	117	136
10	28. viii. 40	130	107
11	6. ix. 40	117	84
12	20. ii. 41	90	110
13	20. ii. 41	104	102
14	19. iii. 41	118	151
15	19. iii. 41	58	94
16	19. iii. 41	98	103
17	19. iii. 41	67	120
18	20. iii. 41	63	100
19	20. iii. 41	82	72
20	20. iii. 41	90	121
21	25. iii. 41	94	97
22	25. iii. 41	104	86
23	25. iii. 41	50	103

The recovery from serum of added vitamin A and carotene is not admissible as evidence of accuracy as there is no relation between the form of vitamin A and carotene already present in the serum and that in which it is added.

There follows a series of values on normal healthy volunteers between 18 and 30 years of age (Table 5). They were all tested on the dark adaptation tester described by the author [Yudkin, 1941] and only those normal by this test were used.

The number of cases is too small to draw any definite conclusions though it is of interest to note that those estimated in February and March are in general lower than those done in the autumn, a possible seasonal effect. The range in general is similar to that of Lindquist [1938] and Kimble [1939].

SUMMARY

The methods of estimating vitamin A and carotene in human plasma are discussed. The reasons for using a photoelectric colorimeter for the measurement of the colours are given.

A parallel series of results obtained by the use of four different methods of extraction shows that the simple extraction from serum or plasma precipitated with alcohol is the most satisfactory.

A preliminary series of normal readings is given.

I would like to thank the many students and others who offered themselves for the experiments, and Prof. C.R. Harington for his kind interest and for the laboratory and other facilities for this work.

REFERENCES

Chevallier & Choron (1935). *C.R. Soc. Biol., Paris*, **118**, 889.
Clausen & McCoord (1938). *J. Pediat.* **13**, 635.
De Haas & Meulemans (1938). *Geneesk. Tijdschr. Ned.-Ind.* **78**, 847.
Dost (1937). *Klin. Wschr.* **16**, 273.
Kimble (1939). *J. Lab. clin. Med.* **24**, 1055.
Koehn & Sherman (1940). *J. biol. Chem.* **132**, 527.
Lanzing (1938). *Geneesk. Tijdschr. Ned.-Ind.* **78**, 3135.
Lindquist (1938). *Acta. med. scand.* **97**, Supplement.
May, Blackfan, McCreary & Allen (1940). *Amer. J. Dis. Child.* **59**, 1167.
Mead, Underhill & Coward (1939). *Biochem. J.* **33**, 589.
Noteyarp & Weedon (1936). *Biochem. J.* **30**, 1705.
Pett & Le Page (1940). *J. biol. Chem.* **132**, 585.
Schneider & Widmann (1935). *Klin. Wschr.* **14**, 670.
Van Eekelen & Emmerie (1934). *Acta. brev. neerl. Physiol.* **4**, 171.
Willstaedt & With (1938). *Nord. med. tidskr.* **15**, 648.
Yudkin (1941). *Brit. J. Ophthal.* **24**, 231.

58. STUDIES IN DETOXICATION

10. THE CONJUGATION AND OXIDATION OF *p*-HYDROXYBENZENESULPHONAMIDE IN THE RABBIT. THE CHARACTERIZATION OF *p*-SULPHONAMIDOPHENYLGLUCURONIDE

By H. G. SAMMONS,[1] JEAN SHELSWELL[1] and R. TECWYN WILLIAMS

From the Physiology Department, Medical School, Hospitals Centre, Birmingham, 15

(*Received 5 March 1941*)

Investigations in progress in this Laboratory on the fate of sulphonamide drugs in the animal body indicate that these are oxidized to a small extent with the production of phenolic substances which may be excreted in conjugation with sulphuric and glucuronic acids [cf. Shelswell & Williams, 1940; Thorpe & Williams, 1940; 1941; Thorpe *et al.* 1941]. In addition to this Scudi [1940] has isolated from the urine of dogs treated with sulphapyridine, the conjugated glucuronide of a hydroxysulphapyridine. It therefore became necessary to study the fate of an aryl sulphonamide containing a phenolic hydroxyl group, since such a study might be of some help in identifying the phenolic substances occurring in urine after the ingestion of sulphanilamide and related drugs. *p*-Hydroxybenzenesulphonamide (hereafter called phenol-*p*-sulphonamide) was selected as it was easily prepared and was also the simplest compound of the type required for study. No previous study has been made of its fate in the animal body, but Buttle *et al.* [1936], among others, have shown that it is therapeutically inactive.

The conjugation and oxidation of phenolsulphonamide

The present work shows that this sulphonamide when fed to rabbits is mainly excreted in the urine conjugated with glucuronic and sulphuric acids; in addition to this a very small portion is oxidized to a catechol derivative, possibly catecholsulphonamide. No free phenolsulphonamide was detected in the urine, since ether extraction of the urine as such (faintly alkaline in reaction) and after acidification gave no trace of the sulphonamide. After hydrolysis with HCl, however, phenolsulphonamide together with the catechol compound could easily be extracted with ether.

The amount of phenolsulphonamide excreted conjugated with sulphuric acid depends upon the dose fed, the percentage conjugation falling with increasing dose (see Table 1). At the smallest dose level used (100 mg./kg.) the sulphate conjugation is more than 50 % of the dose, falling to less than 30 % at the highest level (750 mg./kg.). This behaviour recalls that of phenol, whose sulphate conjugation at various dose levels was studied by one of us [Williams, 1938]. Baumann & Herter [1877–8] have shown that the corresponding acid, phenol-*p*-sulphonic acid, produced no increase in ethereal sulphate excretion in the dog.

---

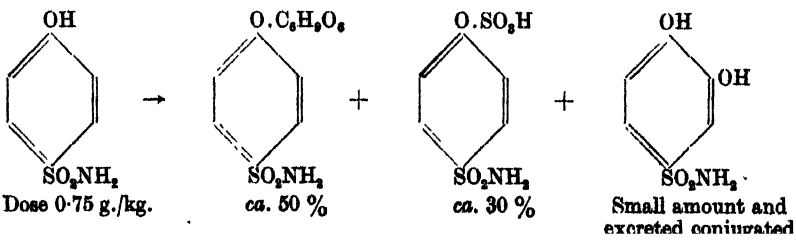
[1] Assisted by grants from the Caroline Harrold Research Fund, University of Birmingham.

Table 2 shows that this is also the case for the rabbit. In addition to phenol-*p*-sulphonic acid (Na salt, B.D.H.) other phenolic sulphonic acids were also fed, namely, 1-hydroxy-2-amino- (B.D.H.), 1-amino-2-hydroxy- [Friedländer, 1911] and 1-amino-3-hydroxy-benzene-4-sulphonic [Friedländer, 1896] acids. None of these acids caused the formation of ethereal sulphate in the rabbit (see Table 2), although they possess a phenolic OH group. A similar result was obtained by Williams [1938] with salicylic acid. Neither was the formation of conjugated glucuronic acid detected (by the naphthoresorcinol reaction) after feeding these acids. Other forms of conjugation were not tested for, as they were very unlikely to occur with the above compounds. It seems very probable that these sulphonic acids are excreted unchanged by the rabbit. Table 2 also includes toluene-*p*-sulphonamide, which causes no increase in ethereal sulphate formation in the rabbit. This compound is of interest since Flaschenträger *et al.* [1934] have shown that about 31 % of it is oxidized to *p*-sulphonamidobenzoic acid in the dog.

Judging from the maximum amount of barium sulphonamidophenylglucuronate that could be isolated from rabbit urine after administration of the sulphonamide, some 50 % is excreted conjugated with glucuronic acid, the dose being 0·75 g./kg. Therefore when the dose is 0·75 g./kg., over 80 % of the phenolsulphonamide can be accounted for by the conjugated glucuronide and ethereal sulphate excreted in the urine.

It was found that crude specimens of the above barium salt gave a green colour with $FeCl_3$ in aqueous solution. This colour was not due to free phenolsulphonamide since the latter gives a feeble purple colour with $FeCl_3$ which is discharged by acid or alkali. The alcoholic filtrates from the preparation of the barium salt (see experimental section) were evaporated to a syrup. The syrup was water-soluble and reduced ammoniacal silver nitrate but not Fehling's solution. Its aqueous solution gave an intense green colour with $FeCl_3$ which turned to blue-green, purple and then red on making the solution progressively more alkaline with Na_2CO_3. Acid discharged the colour. These reactions are similar to those of catechol. Further investigation showed that the syrup was mainly *p*-sulphonamidophenylglucuronide containing a small amount of a catechol derivative which was responsible for the $FeCl_3$ test. This catechol compound was excreted in conjugation since the urine itself gave no $FeCl_3$ reaction nor could a $FeCl_3$ reaction be obtained on ether extracts of the urine at acid or faintly alkaline reactions. The substance could be extracted together with phenolsulphonamide from acid-hydrolysed urines. Several attempts were made to isolate and identify this compound, but owing to the smallness of the amount present, the attempts have so far been fruitless. Such attempts, however, are being continued in conjunction with other work of a similar nature. The very characteristic $FeCl_3$ test and the reduction of ammoniacal $AgNO_3$ leave no doubt in our minds that a catechol derivative is present.

The fate of phenol-*p*-sulphonamide in the rabbit can therefore be summarized as follows:

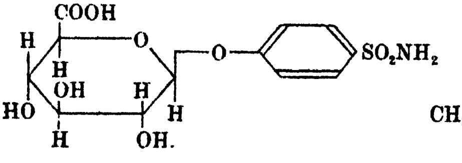


Dose 0·75 g./kg. — *ca.* 50 % — *ca.* 30 % — Small amount and excreted conjugated

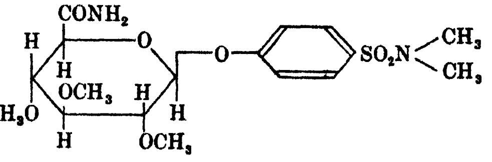
The formation of the above catechol derivative is an example of biological oxidation of the benzene ring in the *o*-position to an existing —OH. Baumann & Preusse [1879] have shown that this *ortho* oxidation takes place during the oxidation of phenol in the dog, since they found small amounts of catechol and quinol in the urine after larger doses of phenol. Biological oxidation can also take place *ortho* to an —NH_2, since *o*-aminophenols are produced in animals from a number of aromatic amino-compounds, e.g. formanilide [Kleine, 1896–7], acetanilide and *o*-acetotoluidide [Jaffe & Hilbert, 1888] and dimethylaniline [Horn, 1936]. There is also the case of β-naphthylamine which is oxidized in rabbits to β-amino-α-naphthol [Wiley, 1938]. A similar oxidation is possible with sulphanilamide [cf. Thorpe *et al.* 1941], since the formation of a catechol derivative from phenolsulphonamide suggests that biological oxidation can take place not only in a position *ortho* to the —OH group but also *meta* to the sulphonamide group.

The characterization of p-*sulphonamidophenylglucuronide*

Removal of barium from barium sulphonamidophenylglucuronate gave the free conjugated glucuronic acid as a brown uncrystallizable syrup. Many phenolic glucuronides behave in this manner and they have often been characterized as barium salts. These salts, however, are usually amorphous and difficult to purify. We therefore subjected the above barium salt to methylation with methyl sulphate and alkali followed by methyl iodide and silver oxide. In this manner the *methyl ester* of *trimethyl* p-*sulphondimethylamidophenylglucuronide* was obtained as a syrup (the sulphonamide group being methylated as well as the sugar residue). Treatment of this ester with methyl alcoholic ammonia gave a crystalline *amide* (II) whereby the glucuronide (I) could be characterized:



(I)



(II)

Pryde & Williams [1933] have shown that, in the case of biosynthetic bornylglucuronide, the sugar ring is of the pyranoid type and, since the glycosidic link in biosynthetic glucuronides is of the β-type (for literature see Pryde & Williams [1934]), the glucuronide excreted by rabbits after administration of phenolsulphonamide appears to be *p*-sulphonamidophenyl-β-*d*-glucopyranuronoside (I).

EXPERIMENTAL

Phenol-*p*-sulphonamide was easily prepared by diazotization of sulphanilamide followed by heating of the acid diazo solution on a boiling water bath. The sulphonamide was extracted from the solution with ether. The yield was 38 g. from 50 g. sulphanilamide. The M.P. after recrystallization from water was 176° (lit. M.P. 176°).

The ethereal sulphate conjugation

The percentage of phenolsulphonamide excreted as ethereal sulphate was determined exactly as described in previous papers on the sulphate conjugation of substituted phenols and sulphanilamide [Williams, 1938; Shelswell & Williams, 1940]. The results are given in Table 1.

Table 1.* *The conjugation of phenolsulphonamide with sulphate in the rabbit*

		Amount of phenolsulphonamide conjugated with sulphate		
Rabbit no.	Dose mg./kg.	%		mg./kg.
77	100	61·1	av. 53	53
11		59·1		
9		47·5		
76		44·9		
9	250	51·5	av. 44	110
63		42·5		
62		38·1		
9	500	38·3	av. 33	165
76		34·9		
70		26·8		
9	750	30·2	av. 27	203
77		29·7		
63		25·5		
76		23·9		

* The figures in this table will include any sulphate conjugated with the catechol derivative.

Table 2

Compound	Dose g./kg.	Sulphate conjugation
1-Hydroxybenzene-4-sulphonic acid	0·5	0
1-Hydroxy-2-aminobenzene-4-sulphonic acid	0·5	0
1-Amino-2-hydroxybenzene-4-sulphonic acid	0·5	0
1-Amino-3-hydroxybenzene-4-sulphonic acid	0·5	0
Toluene-*p*-sulphonamide	0·1	0

The glucuronic acid conjugation

Barium p-*sulphonamidophenylglucuronate.* Rabbits (2–3 kg.) were each given by stomach tube 2 g. of phenolsulphonamide dissolved in 10–20 ml. warm water. The urine was collected during 2 days. It was made faintly acid with glacial acetic acid and then treated with saturated normal lead acetate solution until precipitation was complete. The solution was filtered and, after neutralization by conc. ammonia, the clear yellow filtrate was completely precipitated with saturated basic lead acetate. The basic lead precipitate was collected on a Büchner funnel, thoroughly washed with water, and, before it had time to dry, was made into a thin cream with water. This suspension was gassed with H_2S and the PbS filtered off under suction. The filtrate was concentrated *in vacuo* to a dark syrup which failed to crystallize. The syrup was now dissolved in water and treated with an excess of $BaCO_3$ and the resulting mixture filtered and washed with water. The filtrate and washings (the volume was kept as small as possible) were now poured into a large volume of alcohol, thereby precipitating the required *barium salt.* Next day the salt was filtered, washed with alcohol or acetone and dried in a desiccator. The alcoholic filtrates from this salt were preserved for further investigation (see below). Purification of the salt was accomplished by repeated precipitation from aqueous solution by alcohol, followed by washing with acetone and drying off the acetone *in vacuo.* The barium salt so obtained was a faintly coloured electrostatic powder, soluble in water, giving a pale reddish-brown solution and an intense naphthoresorcinol reaction. It showed $[\alpha]_D -50^\circ$ ($c=1$, in water). (Found: Ba, 16·6; N, 3·1 %. $(C_{12}H_{14}O_9NS)_2Ba$ requires Ba, 16·5; N, 3·3 %.) The yield of the crude salt was 1–1·25 g./g. of phenolsulphonamide fed. Aqueous solutions of the crude salt gave an intense green colour with $FeCl_3$, but the pure compound gave no colour. Removal of the Ba and subsequent suitable treatment gave the free glucuronide as a dark hard resin.

Hydrolysis of the barium salt. The salt (1 g.) was dissolved in 10 ml. water and the barium quantitatively removed with 2*N* H_2SO_4. To the filtrate and washings (40 ml.) from the $BaSO_4$, 10 ml. conc. HCl were added, thus making the solution about 2·5 *N* with respect to HCl. The solution was now gently boiled under reflux for 3 hr. and, after cooling and filtering to remove some black insoluble material, the strongly reducing solution was exhaustively extracted with ether. Removal of the ether left a sticky crystalline residue. Some of the crystals were separated and recrystallized from water. They were phenolsulphonamide, M.P. 170° (not depressed by authentic material, M.P. 176°) and gave the weak purple colour with $FeCl_3$ characteristic of the sulphonamide. The rest of the residue was dissolved in Na_2CO_3 solution and acetic anhydride added with cooling. After a short while, p-*acetoxybenzenesulphonamide* separated and was recrystallized (needles, M.P. 158°)[1] from 50 % aqueous alcohol. Since this acetyl derivative has not been previously described an authentic specimen was prepared. It formed long needles from 50 % aqueous alcohol, M.P. 159°, not depressed by the compound obtained through hydrolysis of the barium salt. (Found: N, 6·7 %. $C_8H_9O_4NS$ requires N, 6·5 %.) Another sample of the barium salt was hydrolysed and the crystalline residue obtained by ether extraction was benzoylated with benzoyl chloride and NaOH. A gum was obtained which, on crystallization from spirit, gave *p*-benzoyloxybenzenesulphonamide, M.P. 237°, as white plates. It did not depress the M.P. of an authentic sample, M.P. 238°. (Schreinemakers [1897] gives M.P. 234–236°.)

Methylation of the barium salt. The barium salt (5 g.) was dissolved in 50 ml. water and acetone was added until a slight permanent precipitate was obtained. The solution was then methylated at 35° with 25 ml. methyl sulphate and 60 ml. 30 % NaOH added dropwise with stirring. The stirring was continued for 5 hr. with occasional additions of acetone and then at 70° to destroy excess methyl sulphate and remove acetone. The solution was now cooled in ice, neutralized with 5*N* H_2SO_4 and made slightly acid to Congo red. Methylated spirit was added to precipitate Na_2SO_4 which was filtered under suction and thoroughly washed with spirit. The filtrate and washings were made slightly alkaline with dilute NaOH and concentrated to a thin syrup. The latter was now taken up in a small amount of 5*N* H_2SO_4 and the sticky precipitate formed was exhaustively extracted with chloroform. The chloroform solution was washed with water, dried over anhydrous Na_2SO_4 and then concentrated to an acid syrup (2·76 g.) which failed to crystallize. The syrup was now given three treatments with methyl iodide and silver oxide, i.e. until a constant methoxyl value was obtained. A yellow viscous syrup (2·2 g.) was obtained which could not be crystallized. This is presumably the *methyl ester* of 2:3:4-*trimethyl* p-*sulphondimethylamidophenylglucuronide*. It showed $[\alpha]_D^{21^\circ} -51{\cdot}3^\circ$ ($c=1$, in $CHCl_3$). (Found: OMe, 29·4 %. $C_{18}H_{27}O_9NS$ requires OMe, 28·6 %.)

2:3:4-Trimethyl p-*sulphondimethylamidophenylglucuronamide*. The above methyl ester (0·5 g.) was dissolved in 5 ml. dry methyl alcohol and the solution saturated with dry ammonia at 0°. After 20 hr. in a desiccator at room temp. the solvent was evaporated and the residue crystallized on the addition of ether.

---

[1] Melting points are uncorrected.

The *amide* was recrystallized from acetone-ether. It formed long needles, M.P. 154–5°, slightly soluble in ether, but soluble in warm water, alcohol and acetone (yield 90 % theory). It showed $[\alpha]_D^{21°} -42·3°$ ($c=0·94$ in abs. alcohol) and $[\alpha]_D^{20°} -52·2°$ ($c=1$, in water). (Found: C, 48·6; H, 6·1; N, 7·0; OMe, 23·1. $C_{17}H_{26}O_8N_2S$ requires C, 48·8; H, 6·2; N, 6·7; OMe, 22·25 %.)

Experiments with the oxidation product of phenolsulphonamide

The alcoholic filtrates and washings from the preparation of the barium salt were concentrated *in vacuo* to a syrup. This syrup was dissolved in absolute alcohol, leaving behind a quantity of the barium salt which had escaped precipitation in the original preparation. Three concentrations and solutions in alcohol were necessary to remove all the barium salt; finally, a syrup completely soluble in alcohol was obtained. The amount of this syrup was 1–1·5.g. from six rabbits fed with 2 g. each of phenolsulphonamide. The syrup was soluble in water and its solution did not reduce Fehling's solution, but reduced ammoniacal silver nitrate vigorously on warming. It gave the typical catechol $FeCl_3$ reaction (green, blue-green, purple, red on increasing the alkalinity) and gave an intense naphthoresorcinol reaction for glucuronic acid. One sample was methylated with methyl sulphate and alkali and the product extracted with ether. The ethereal solution was evaporated to a thick liquid and distilled. A brownish distillate was obtained, but no veratrole (dimethyl catechol) could be detected in it and the nature of the distillate was not discovered. Another methylated sample was subjected to treatment with various solvents in an attempt to crystallize out veratrole-4-sulphonamide, an authentic sample [Paul, 1906] of which had been prepared for comparison. No veratrolesulphonamide was obtained. Another sample of the syrup (1·5 g.) was hydrolysed by boiling under reflux with 20 ml. of 2*N* HCl. The hydrolysate was then extracted with ether in a continuous extractor. On evaporation of the extract a brownish crystalline residue was obtained which gave an intense green colour with $FeCl_3$. Treatment of this residue with Na_2CO_3 and acetic anhydride produced *p*-acetoxybenzenesulphonamide. The isolation of this compound, together with the fact that the hydrolysate was very strongly reducing, indicated that the syrup was mainly *p*-sulphonamidophenylglucuronide, containing a small amount of the catechol derivative. The isolation of the latter is now being tackled by other methods in conjunction with other work of a similar nature in this Laboratory.

SUMMARY

The fate of *p*-hydroxybenzenesulphonamide (phenol-*p*-sulphonamide) in the rabbit has been studied. At a dose level of 0·75 g./kg. about 50 % of the dose is excreted conjugated with glucuronic acid, about 30 % with sulphuric acid and a small amount is oxidized to a catechol derivative, probably catecholsulphonamide. No free phenolsulphonamide was excreted.

A study was also made of the ethereal sulphate conjugation of phenolsulphonamide at various dose levels and it was found that the percentage conjugated fell with increasing dose.

The conjugated glucuronide of phenolsulphonamide was isolated as a barium salt and characterized by means of the crystalline amide of the 2:3:4-trimethyl-*p*-sulphondimethylamidophenyl-β-*d*-glucuronide.

It has been shown that phenol-*p*-sulphonic acid, 1-hydroxy-2-amino-, 1-amino-2-hydroxy- and 1-amino-3-hydroxy-benzene-4-sulphonic acids do not conjugate with glucuronic and sulphuric acids in the rabbit and are probably excreted unchanged.

REFERENCES

Baumann & Herter (1877–8). *Hoppe-Seyl. Z.* **1**, 244.
—— & Preusse (1879). *Hoppe-Seyl. Z.* **3**, 156.
Buttle, Gray & Stephenson (1936). *Lancet*, **1**, 1289.
Flaschenträger, Bernhard, Löwenberg & Schläpfer (1934). *Hoppe-Seyl. Z*, **225**, 157.
Friedländer (1896). *Fortschr. Teerfarb.* **3**, 59.
—— (1911). *Fortschr. Teerfarb.* **9**, 148.
Horn (1936). *Hoppe-Seyl. Z.* **238**, 84.
Jaffe & Hilbert (1888). *Hoppe-Seyl. Z.* **12**, 295.
Kleine (1896–7). *Hoppe-Seyl. Z.* **22**, 327.
Paul (1906). *Ber. dtsch. chem. Ges.* **39**, 2773.
Pryde & Williams (1933). *Biochem. J.* **27**, 1197.
—— —— (1934). *Biochem. J.* **28**, 133.
Schreinemakers (1897). *Rec. Trav. chim. Pays-Bas*, **16**, 423.
Scudi (1940). *Science*, **91**, 486.
Shelswell & Williams (1940). *Biochem. J.* **34**, 528.
Thorpe & Williams (1940). *Nature, Lond.*, **146**, 686.
—— —— (1941). *Biochem. J.* **35**, 61.
—— —— & Shelswell (1941). *Biochem. J.* **35**, 52.
Wiley (1938). *J. biol. Chem.* **124**, 627.
Williams (1938). *Biochem. J.* **32**, 878.

Note added 27 May 1941. The catechol derivative mentioned above has now been identified as catechol-4-sulphonamide, since suitable treatment, including methylation, of ether extracts of the hydrolysed urine gave crystalline *veratrole-4-sulphondimethylamide*, M.P. 105°, identical with a synthetic sample. Synthetic *catechol-4-sulphonamide* has also been prepared and found to be a non-crystalline resin, although *catechol-4-sulphonanilide* is crystalline, M.P. 221°. A description of this work will be reported when completed.

59. THE HYDROLYTIC DEGRADATION OF THE ANTIGENIC COMPLEX OF *BACT. TYPHOSUM* Ty2

By GEORGE GROSVENOR FREEMAN[1] and
THOMAS HEMINGWAY ANDERSON

*From the Division of Biochemistry, London School of Hygiene
and Tropical Medicine, University of London*

(*Received 13 March 1941*)

The antigens of a number of Gram-negative micro-organisms have been isolated in a relatively pure condition and apparently unchanged in their specific immunological properties by various methods in the work of Boivin *et al.* [1933; 1934; 1935; 1937], Raistrick & Topley [1934], Topley *et al.* [1937], Morgan [1937], Morgan & Partridge [1940], and Miles & Pirie [1939, 1, 2, 3]. By use of the trichloroacetic acid method of extraction of Boivin *et al.* and the tryptic digestion method of Raistrick & Topley, Freeman *et al.* [1940] obtained the antigens of *Bact. typhosum* and *Bact. typhi-murium* and showed that by growth of the organisms in a synthetic medium various sources of contamination were eliminated.

The present communication describes a more detailed investigation of the chemical nature of the antigenic fractions obtained from *Bact. typhosum* Ty2 by the above methods.

The Ty2 strain of *Bact. typhosum* and its isolated antigen react with both O and Vi antisera; this is regarded by some authors as being due to the presence of two chemically and immunologically distinct entities, the O and Vi antigens. Boivin & Mesrobeanu [1938, 2] claim to have separated these components by precipitating the Vi antigen with uranyl acetate but attempts to repeat this work by the present authors have so far proved unsuccessful. Malék [1939] was also unable to separate the O and Vi component antigens by means of uranyl acetate. Meyer [1938] has, however, stated that the O and Vi antigens of an O+Vi strain of *Bact. typhosum* are distinct molecular entities which can be separated by their precipitation reactions towards specific antisera. Thus, whilst in the investigation to be described the antigen of *Bact. typhosum* Ty2 has been regarded as an individual molecule, the possibility of its consisting of component O and Vi antigenic molecules must not be overlooked.

EXPERIMENTAL

The bulk of the starting material in this investigation was prepared from the dried cells of *Bact. typhosum* Ty2, grown on a synthetic medium, by tryptic digestion as described by Freeman *et al.* [1940].

Properties of the antigenic complex

The tryptic digest antigen of *Bact. typhosum* Ty2 dissolves readily in water giving opalescent solutions even when the concentration of antigen is as low as

---

[1] Grocers' Company Scholar for Medical Research.

0·4 %. The bulk of the substance remains in solution after 30 min. centrifuging at 15,000 r.p.m. The antigenic complex also dissolves readily in formamide and formic acid giving solutions which are practically free from opalescence, but it is insoluble in glacial acetic acid, dioxan and in concentrated aqueous solutions of phenol. Qualitative tests have been made on 0·5 ml. quantities of a 0·4 % aqueous solution of the antigen; the test solutions were centrifuged at about 2000 r.p.m. for 15 min. and 0·5 ml. of the opalescent supernatant solution was mixed with 0·5 ml. of the reagent. Acid potassium permanganate was slowly reduced by the antigenic solution in the cold and more rapidly on warming. Addition of uranyl acetate solution produced an immediate flocculent precipitate, whilst phosphotungstic acid in 5 % H_2SO_4 gave a precipitate on standing and neutral lead acetate an increased opalescence on standing. No precipitates were obtained on addition of Esbach's reagent, 10 % trichloroacetic acid solution, salicylsulphonic acid or tannic acid. Tyrosine was present as indicated by positive Millon's, xanthoproteic, α-nitroso-β-naphthol and Ehrlich's diazo reactions. The Adamkiewicz and aldehyde reactions and Ehrlich's *p*-dimethylaminobenzaldehyde test gave negative results showing the absence of tryptophan. The sodium fusion test and boiling of the antigen with strong NaOH and addition of lead acetate proved the absence of sulphur. A positive Sakaguchi reaction for arginine was obtained. The antigenic solution also gave positive biuret and ninhydrin reactions, indicating the presence of peptide linkages and amino-groups respectively. The substance gave a very strong Molisch's reaction but did not reduce Fehling's solution. Pentose residues (furfuraldehyde, Tollen's phloroglucinol and Bial's tests), ketose (Seliwanoff's and Foulger's tests) and glucuronic acid residues (Tollen's naphthoresorcinol reaction) were found to be absent. The solution gave no iodine reaction.

The nitrogen content and reducing sugar value of the antigenic preparations varied within narrow limits from batch to batch and a typical sample gave: N, 7·2 %; reducing sugar value, 32 %; $[\alpha]_{5461} = +104^\circ$ (in aqueous solution, $c=1$), $[\alpha]_D^{17^\circ} = +69^\circ$ (in formamide solution, $c=1$), and $[\alpha]_D^{18^\circ} = +68^\circ$ (in formic acid solution, $c=1$); non-volatile ash, 12·2 %; organic P, 3·0 %; inorganic P, 1·2 % (N/organic P ratio, 5·0). Determination of amino-N by Van Slyke's method showed that 0·6 % of N was present as amino-groups.

The tryptic digest antigen was toxic for mice, having an average lethal dose of 0·3 mg. [Freeman *et al.* 1940]. Immunizing experiments similar to those described by Topley *et al.* [1937] have been carried out on practically every batch of antigen, and the results have shown that the substance possesses a high degree of immunological activity comparable with that of the original dried bacterial cells.

Acetic acid hydrolysis of the antigenic complex

The tryptic digest antigen was dispersed by shaking with 0·1*N* acetic acid to give a 1 % solution, which was centrifuged at 15,000 r.p.m. for 2–3 hr. to remove undissolved material ('undissolved residue'). The supernatant solution which was slightly opalescent was hydrolysed at 100° on the boiling water bath for 12 hr. On heating, the solution first clarified a little (30 min.), but after 2 hr. it became very opalescent and an insoluble substance began to flocculate out. After about 8 hr. heating the flocculation appeared to be complete and there was no further deposition of precipitate and no change of optical rotation during the last 2 hr. The insoluble material ('hydrolysis precipitate') was centrifuged off, washed with a little water and dried *in vacuo* over phosphorus pentoxide.

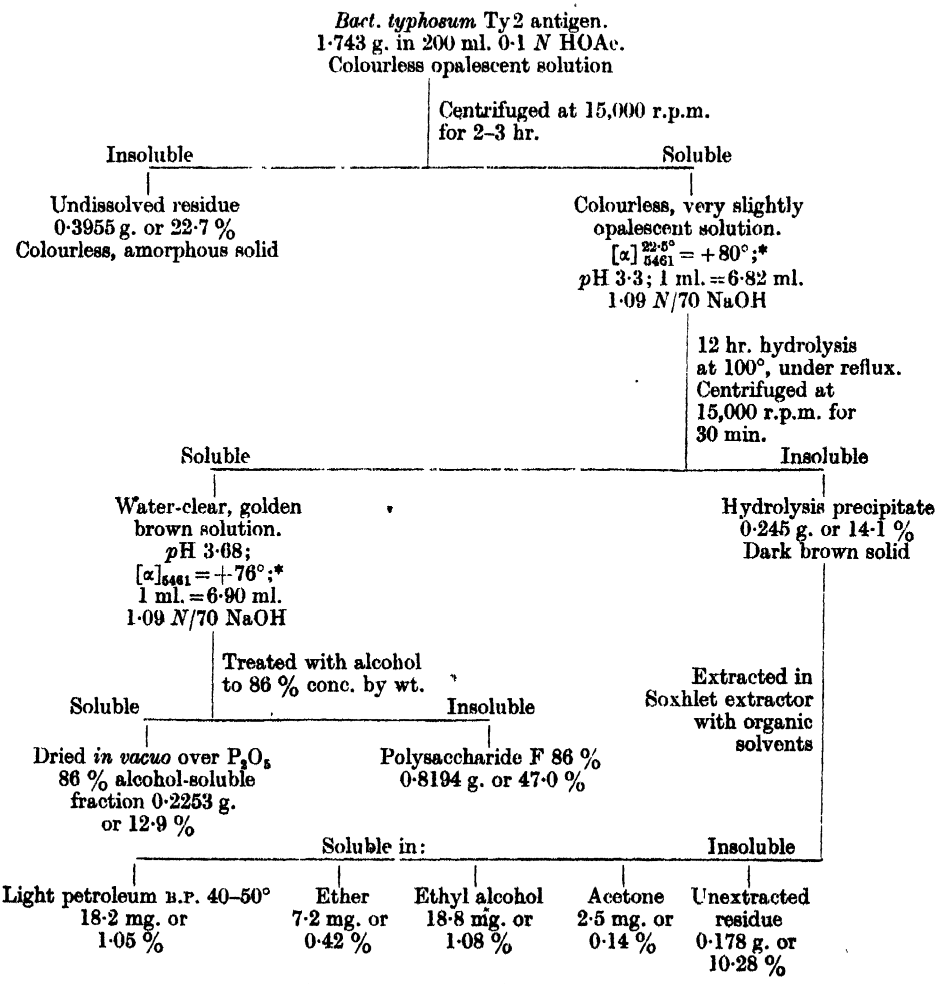
The dried hydrolysis precipitate was then exhaustively extracted in a Soxhlet extractor, successively with:

(1) Light petroleum, B.P. 40–50°,
(2) Pure anhydrous ether,
(3) Absolute ethyl alcohol, and
(4) Acetone.

The solvents were evaporated from the extracted lipid components, which were stored *in vacuo*. The remainder ('unextracted residue') was dried *in vacuo* over phosphorus pentoxide.

The acetic acid supernatant solution, containing the soluble hydrolysis products, was water-clear and golden brown in colour; it was treated with sufficient absolute alcohol to bring the alcoholic concentration to 86%, by weight, giving a precipitate rich in polysaccharide ('Polysaccharide F 86 %'). This

Table 1. *0·1*N *acetic acid hydrolysis of* Bact. typhosum *Ty2 'tryptic digest antigen'*


Bact. typhosum Ty2 antigen.
1·743 g. in 200 ml. 0·1 *N* HOAc.
Colourless opalescent solution
Centrifuged at 15,000 r.p.m. for 2–3 hr.
Insoluble
Soluble
Undissolved residue
0·3955 g. or 22·7 %
Colourless, amorphous solid
Colourless, very slightly opalescent solution.
$[\alpha]_{5461}^{22\cdot5^\circ} = +80^\circ$;*
*p*H 3·3; 1 ml. = 6·82 ml. 1·09 *N*/70 NaOH
12 hr. hydrolysis at 100°, under reflux. Centrifuged at 15,000 r.p.m. for 30 min.
Soluble
Insoluble
Water-clear, golden brown solution.
*p*H 3·68;
$[\alpha]_{5461} = +76^\circ$;*
1 ml. = 6·90 ml. 1·09 *N*/70 NaOH
Hydrolysis precipitate
0·245 g. or 14·1 %
Dark brown solid
Treated with alcohol to 86 % conc. by wt.
Soluble
Insoluble
Extracted in Soxhlet extractor with organic solvents
Dried *in vacuo* over P_2O_5
86 % alcohol-soluble fraction 0·2253 g. or 12·9 %
Polysaccharide F 86 %
0·8194 g. or 47·0 %
Soluble in:
Insoluble
Light petroleum B.P. 40–50°
18·2 mg. or 1·05 %
Ether
7·2 mg. or 0·42 %
Ethyl alcohol
18·8 mg. or 1·08 %
Acetone
2·5 mg. or 0·14 %
Unextracted residue
0·178 g. or 10·28 %

* Uncorrected for ash.

Total recovery of fractions = 96 %.

fraction was washed twice with absolute alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide.

The alcoholic supernatant solution and washings of polysaccharide F 86 % were evaporated to dryness *in vacuo*, giving a brown-coloured sticky residue: '86 % alcohol-soluble fraction'.

The fractionation is summarized in Table 1. The fractions thus isolated have been submitted to qualitative and quantitative examination; Kjeldahl N, non-volatile ash, reducing sugar value, specific optical rotation, total and inorganic P, amino-N and acidity were determined. The N, ash, reducing sugar value and specific rotation determinations were carried out as described by Freeman *et al.* [1940]. Total P determinations were made on 0·5–1·0 mg. quantities of substance by Stewart & Hendry's [1935] modification of the Fiske & Subbarow [1925] method. Inorganic P determinations were made on 2–5 mg. quantities of substance by the Fiske & Subbarow method. Organic P has been taken as the difference between total and inorganic P, and equivalent N/organic P ratios have been calculated on this basis. The amino-N content of the soluble fractions has been determined on 10 mg. samples by the Van Slyke method. Acidity determinations were made by titrating 5–10 mg. quantities dissolved in a few ml. water with 0·014*N* NaOH using phenolphthalein as indicator. Except where otherwise stated analytical results are quoted on an ash-free basis. The results obtained for the fractions isolated by partial acetic acid hydrolysis of the antigenic material are summarized in Table 2.

Table 2. *Analytical data of fractions isolated from* Bact. typhosum *Ty2 antigen* (1·74 g.

	Un-dissolved residue	Un-extracted residue	Total lipid	Poly-saccharide F 86 %	86 % alcohol-soluble fraction
Weight (g.)	0·395	0·178	0·047	0·819	0·225
Yield (%)	22·7	10·3	2·7	47·0	12·9
N (%)	8·9	6·0	0·6	3·6	13·4
Non-volatile ash (%)	7·4	5·0	—	20·2	5·4
Reducing sugar value (%)	28	10	—	55	19
$[\alpha]_{5461}$			—	+142°	+7°
Inorganic P (%)			—	3·0*	0·2*
Organic P (%)	4·1	2·2	0·5	1·6	0·6
N/org. P ratio	4·7	6·0	2·6	4·8	48
Amino-N (%)			—	0·5	3·0
Acidity ml. *N* NaOH/g.			—	0·55*	1·38*
% Yield on total dissolved matter	—	13·3	3·5	60·8	16·7

* Uncorrected for ash.

Further samples of antigenic material were hydrolysed under slightly different conditions with similar results. Soru & Combiesco [1940] report fission of the *Bact. typhosum* O-antigen, prepared by trichloroacetic acid extraction of the organisms, by hydrolysis with 0·04*N* acetic acid for 45–60 min. at 100°. Some of our hydrolyses were made with 0·1*N* acetic acid for 2 hr., and in one experiment a trichloroacetic acid extract of the bacteria, 'Boivin antigen F 68 %', was hydrolysed with 0·04*N* acetic acid for 60 min. at 100°. In these cases in which a shorter period of hydrolysis was used the flocculation of the hydrolysis precipitate was incomplete and this fraction could not be completely precipitated on centrifuging. Addition of alcohol to about 30 % concentration, by weight, was required to complete the flocculation of the hydrolysis precipitate. (The polysaccharide component is completely precipitated between the limits

Table 3. *Hydrolysis of further batches of* Bact. typhosum *Ty2 antigen with dilute acetic acid*

Conditions of hydrolysis, etc.		Hydrolysis precipitate		Polysaccharide F 86 %	86 % alcohol-soluble fraction
1. 3·644 g. tryptic digest antigen dissolved in 335 ml. 0·1 *N* acetic acid and hydrolysed at 100° for 12 hr.	Yield g. ...	0·493		1·937	0·498
	% of antigen ...	13·6		53·2	13·7
	Properties ...	Main fraction after solution in *N* NaOH and repptn. by HCl and extraction with organic solvents gave: N 8·2 %, R.V. 10 %, ash 2·0 %, P (total) 2·4 %		After two fractionations between 42 % and 85 % conc. of alcohol, by weight, gave: N 2·2 %, R.V. 68 %, ash 6·9 %	P (total) 1·9 % Ash 9·0 % R.V. 19 % N 14·3 % Amino-N 1·8 %
2. 5·07 g. tryptic digest antigen dissolved in 500 ml. 0·1 *N* acetic acid and hydrolysed at 100° for 12 hr.	Yield g. ...	1·122		2·602	1·157
	% of antigen ...	22·8		53·1	23·6
3. 4·54 g. tryptic digest antigen dissolved in 500 ml. 0·1 *N* acetic acid and hydrolysed at 100° for 2 hr.	Yield g. ...	1·1576		2·721	0·2926
	% of antigen ...	25·5		59·9	6·4
4. 0·598 g. 'Boivin antigen F 68 %' dissolved in 112·5 ml. 0·04 *N* acetic acid and hydrolysed at 100° for 60 min.		Ppt. on centrifuging	Alcoholic ppt. F 50 %	(F 50–85 %)	—
	Yield g. ...	0·0156	0·3092	0·1828	—
	% of antigen ...	2·6	51·7	30·6	—
	Properties ...	N 6·0 %	N 6·4 % Ash 14·8 %	R.V. 64 % Ash 8·1 % N 2·57 % P (total) 1·8 %	—

of 40–85 % concentration of alcohol, by weight, but does not precipitate to any appreciable extent at 30 % alcoholic concentration.) These experiments are summarized in Table 3.

The undissolved residue which may be separated from solutions of the antigenic material by high-speed centrifuging, as in the original hydrolysis experiment, is to be regarded as an insoluble or 'denatured' modification of the antigen, which tends to pass into an insoluble form on drying and storage. This fraction, therefore, has not been removed in the later experiments but submitted to hydrolysis with the bulk of the antigenic material.

The experiments 1–3, summarized in Table 3, confirm the results of the original hydrolysis experiment (Tables 1 and 2); when the time of hydrolysis is shortened to 2 hr. (exp. 3) the separation into soluble and insoluble fractions was much less definite and the hydrolysis precipitate was not completely flocculated until an alcoholic concentration of 32 % (by weight) had been reached. 6 % of insoluble substance was removed by centrifuging at 3000 r.p.m. on an angle centrifuge for 4 hr., leaving a very opalescent supernatant solution, from which the remaining 19·5 % of insoluble matter was precipitated by an alcoholic concentration of 32 % (by weight) leaving a water-clear supernatant solution. In this experiment the 86 % alcohol-soluble fraction was also much smaller in amount. In exp. 4, in which a trichloroacetic acid extract of *Bact. typhosum* Ty2 'Boivin antigen F 68 %') was hydrolysed under the conditions employed by Soru & Combiesco [1940], there was an even greater difficulty in flocculating the insoluble fraction. Only 2·6 % could be precipitated by centrifuging (3000 r.p.m. on an angle centrifuge for 3 hr.), and alcohol to 50 % concentration (by weight) had to be added in order to complete the flocculation of the very opalescent supernatant solution. This produced 52 % of substance, which was probably incompletely hydrolysed. A crude polysaccharide, alcoholic fraction F 50–85 %, was obtained which was very similar in properties to the crude polysaccharide obtained on hydrolysis of the tryptic digest antigen.

The mean of the results obtained in exps. 1–3 and in the original hydrolysis experiment (Table 2) point to the conclusion that on mild hydrolysis the antigen of *Bact. typhosum* Ty2 yields about 57 % of crude polysaccharide, 18 % of alcohol-soluble fraction and 20 % of hydrolysis precipitate, of which about 4 % (in terms of the original weight of antigen) is of a lipid nature. These four constituents will now be considered in fuller detail.

The polysaccharide component of the antigenic material

The crude polysaccharide was a water-soluble, almost colourless amorphous substance, which corresponded to about 57 % of the antigenic complex. As first precipitated it contained 3·6 % N, 1·6 % organic P and a considerable amount of ash. On hydrolysis with 2*N* HCl for 2 hr. in a sealed tube 55 % of reducing sugars were liberated (Hagedorn & Jensen). At this stage the polysaccharide had a small titratable acidity corresponding to 0·5 ml. *N* NaOH/g. and had a high specific rotation of about $[\alpha]_{5461} + 140^\circ$. The polysaccharide dissolved readily in water, giving solutions which were water-clear. Solutions of the crude material failed to give a positive Millon's reaction or α-nitroso-β-naphthol test for tyrosine; the biuret reaction was also negative. Weakly positive ninhydrin tests and the Sakaguchi reaction for arginine indicated that small amounts of amino-acid residues remained as impurities in the polysaccharide. This was confirmed by the appearance of a trace of precipitate on addition of phosphotungstic acid in 5 % H_2SO_4 and of an opalescence on addition of neutral lead acetate. The polysaccharide gave a very strongly positive Molisch's reaction. It gave no coloration with iodine and failed to reduce Fehling's solution. There was no evolution of furfuraldehyde on heating with 12 % HCl. Bial's reaction and Tollen's phloroglucinol test for pentoses were also negative as were the Seliwanoff test and Foulger's test for ketoses. Tollen's naphthoresorcinol test indicated the absence of uronic acid residues.

The purification of the polysaccharide by means of alcoholic and acetic acid fractionations or by preparation of the triacetyl derivative and subsequent deacetylation will be described in a later communication. An identical polysaccharide was obtained by treatment of the dried bacterial cells with dilute acetic acid at 100°. After purification the polysaccharide was obtained free from N and contained only traces of P (0·1–0·2 %). The purified substance had a reducing value of 1·1 % before hydrolysis, and on hydrolysis with $2N$ HCl for 2 hr. at 100° in a sealed tube gave 83 % of reducing sugars (Hagedorn & Jensen). It was highly dextrorotatory, $[\alpha]_D = +114°$ and $[\alpha]_{5461} = +140°$. Sulphur was absent and the ash content had been reduced to 1–2 %. On hydrolysis the polysaccharide yielded 3 % of volatile fatty acid (calculated as acetic acid), indicating the presence of acetyl residues in the molecule. (Found: C, 44·41; H, 7·12 %. $C_6H_{10}O_5$ requires C, 44·41; H, 6·22 %.)

The above-mentioned qualitative tests on the crude material indicate the absence of ketose, pentose and uronic acid residues from the polysaccharide molecule and repetition of the tests on the purified substance gave the same results, showing that such residues are either entirely absent or present only in very small amounts. On hydrolysis of the polysaccharide, derivatives of *d*-mannose, *d*-galactose and *d*-glucose have been isolated; these hexoses constitute the bulk of the polysaccharide molecule.

Our colleague, Dr A. Felix, has shown that solutions of the polysaccharide precipitate with 'pure' *Bact. typhosum* O-antiserum to a dilution of 1 in 15,000,000 of the polysaccharide, but not with 'pure' Vi-antiserum. The polysaccharide represents the O-hapten of the bacterium.

The polypeptide component of the antigenic material
(*'unextracted residue'*)

The fraction which is insoluble in water and organic solvents ('unextracted residue') constitutes about 16 % of the antigenic complex. In its crude state it is obtained as a brownish coloured solid which dissolves almost completely in $2N$ NaOH and is reprecipitated on acidification. Identification of constituent residues in the unhydrolysed substance is difficult owing to its insolubility. Although the ninhydrin reaction is negative, the presence of tyrosine and arginine is evident from positive xanthoproteic, Millon's and α-nitroso-β-naphthol tests, and the Sakaguchi reaction respectively. After partial hydrolysis with mineral acid a positive biuret reaction is obtained.

Fractionation of the hydrolysis precipitate. The dark greyish-brown hydrolysis precipitate obtained in exp. 1 (Table 3), and unextracted with organic solvents, was suspended in water and NaOH was added to N concentration. The bulk of the precipitate dissolved in the alkali to give a dark brown solution, leaving a small amount of undissolved residue which was removed by centrifuging. This undissolved residue was suspended in a few ml. of $2N$ NaOH when it showed anisotropy of flow and remained undissolved. It was recovered by acidification as a greyish-white precipitate. This was dried *in vacuo* over P_2O_5 and yielded 0·035 g. of 'hydrolysis precipitate (fraction insoluble in NaOH and HCl)'.

The clear brown alkaline solution containing the bulk of the hydrolysis precipitate was made slightly acid with HCl when a buff-coloured precipitate of the acidic hydrolysis precipitate was obtained. The solid was centrifuged, taken up in a small quantity of 0·1 *N* NaOH in which it dissolved readily, and reprecipitated by neutralization with HCl; the precipitate was finally washed free from chlorides with distilled water.

The golden-yellow acid supernatant solution obtained on reprecipitation of the hydrolysis precipitate was neutralized and concentrated *in vacuo*. The concentrated opalescent suspension was dialysed in a collodion sac against frequent changes of distilled water for 24 hr. to remove sodium chloride, etc. and the contents of the sac were evaporated to dryness *in vacuo* giving 0·107 g. of a brown substance: 'Hydrolysis precipitate (fraction soluble in NaOH and HCl)'.

The bulk of the hydrolysis precipitate (i.e. soluble in NaOH and reprecipitated by HCl) was suspended in 20 ml. water and extracted with ether in a continuous liquid extraction apparatus for 40 hr. The ethereal solution on evaporation yielded 0·125 g. of a light brown waxy substance. The unextracted brown hydrolysis precipitate was centrifuged down and dried *in vacuo* over P_2O_5 yielding 0·290 g. of a brownish-black substance. This was extracted in a Soxhlet extractor with absolute ethyl alcohol for 24 hr., and 0·0238 g. of brown fatty material was recovered from the alcoholic solution. The unextracted residue was then extracted with acetone for 24 hr. in a Soxhlet extractor and 0·009 g. of lipid material was recovered from the acetone extract. The final unextracted residue consisted of 0·193 g. of a dark brown amorphous substance. The above fractionation is summarized in Table 4.

Table 4. *Fractionation of 'hydrolysis precipitate'*

'Hydrolysis precipitate'

Dispersed in
N NaOH

Soluble

Clear dark brown
solution, neutralized
with HCl and
centrifuged

Golden-yellow solution, concentrated,
dialysed and
evaporated to dryness

'Hydrolysis precipitate
soluble in NaOH and HCl'
0·107 g. (3·0 %)

Insoluble

'Hydrolysis precipitate
insoluble in NaOH'
0·035 g. (0·96 %).
Brownish-black substance

Insoluble

Extracted with
organic solvents

Soluble in:

Ether	Alcohol	Acetone	Insoluble
0·125 g. (3·4 %)	0·024 g. (0·7 %)	0·009 g. (0·2 %)	'Unextracted residue' 0·193 g. (5·3 %) Brownish-black coloured substance. N 8·2 %; R.V. 10 %; ash 2·0 %; P (total) 2·4 %

The purified fraction obtained in the above fractionation contains N 8·2 %, P (total) 2·4 % and has a reducing value on hydrolysis with 2 *N* HCl for 2 hr. in a sealed tube at 100° of 10 % apparent glucose. The substance is acidic, being readily soluble in dilute alkalis in the cold, but insoluble in water and dilute acids; it is so resistant to acid hydrolysis that it is only partially soluble on treatment with $2N$ HCl in a sealed tube for 2 hr. at 100°. The partial hydrolysate thus obtained gives an intense yellowish-red coloration with diazobenzene-sulphonic acid in alkaline solution (Ehrlich's diazo-reaction) and a positive α-nitroso-β-naphthol reaction indicating the presence of tyrosine. The ninhydrin reaction is also strongly positive at this stage.

Tryptic digestion of the 'unextracted residue'. The action of trypsin on the polypeptide-like fraction ('unextracted residue') has been investigated and the liberation of amino-acids during tryptic digestion followed by means of the Van Slyke method. 0·075 g. of the 'unextracted residue', prepared as described in Table 4, was freshly precipitated by acidifying an alkaline solution of the substance and then suspended in 15 ml. of borate buffer at *p*H 8·46. A brownish-coloured opalescent suspension was obtained. 0·003 g. commercial trypsin (Fairchild Bros. and Foster, New York) was added and the digest incubated at 37° for 14 days at *p*H 8·2–8·5, a further 0·003 g. trypsin being added after 2 days' digestion. 1 ml. portions of the digest were withdrawn at intervals and the amino-N estimated by the Van Slyke method. A blank determination was also made on a sample of trypsin which itself contained a small amount of amino-N. After 2 days' digestion only 7·3 % of the total N had been liberated as amino-N and 13·4 % after 14 days. The substance was therefore only slowly and very incompletely attacked by trypsin; its resistance to tryptic digestion is evident from the fact that after 14 days' exposure to the enzyme under these conditions an insoluble substance similar to the undigested compound was precipitated on acidification.

Acid hydrolysis of the 'unextracted residue'. Morgan & Partridge [1940] found that the polypeptide component of the *B. dysenteriae* (Shiga) antigenic complex was readily hydrolysed by $10N$ formic acid containing 3·6 % HCl. It has already been pointed out that the polypeptide-like fraction from the *Bact. typhosum* Ty 2 antigenic complex was not completely dissolved by treatment with $2N$ HCl for 2 hr. in a sealed tube at 100°. 0·008 g. quantities of the 'unextracted residue' were treated with 1 ml. $10N$ formic acid containing 3·6 % HCl and hydrolysed in sealed tubes at 100° for various lengths of time. At the end of the periods of hydrolysis the acid hydrolysates were exactly neutralized with NaOH and the amino-N determined by the Van Slyke method. The substance did not dissolve completely in the acid mixture even after 10 hr. hydrolysis. The results are summarized in Table 5.

Table 5. *Rate of increase of amino-N during acid hydrolysis of the 'unextracted residue' of the* Bact. typhosum *Ty 2 antigenic complex*

Weight of sample mg.	Time hr.	Amino-N per ml. mg.	Total N per ml. mg.	Amino-N as % of total N
—	0	Substance insoluble	—	—
8·60	0·5	0·081	0·686	11·8
8·50	2	0·209	0·678	30·8
8·50	3	0·254	0·678	37·4
8·80	5	0·317	0·702	45·2
8·85	6	0·317	0·706	44·9
8·65	10	0·332	0·690	48·1

The results in Table 5 show that during acid hydrolysis the amino-N of the fraction of the antigenic complex (insoluble in acids and organic solvents) increases to 48 % of the total N after 10 hr. hydrolysis, and that at this stage the liberation of amino-acids has reached a maximum. The insoluble nitrogenous component of the antigen contains tyrosine and arginine, it liberates 13·4 % of its total nitrogen as amino-groups on prolonged tryptic digestion, and 48 % of amino-N on acid hydrolysis and must therefore be regarded as a polypeptide.

The 86 % alcohol-soluble fraction of the antigenic complex

This fraction contains N 13·4 %, P (inorganic 0·2 %, organic 0·6 %), and has a small reducing power of 13 % (apparent glucose) which increases to 18 % on hydrolysis with 2*N* HCl in a sealed tube for 2 hr. at 100°. Of the total N, 3·0 % is in the form of amino-groups (Van Slyke). In aqueous solution the substance has a small dextrorotation, $[\alpha]^{20^\circ}_{5461} + 7^\circ$ ($c = 1$). The substance dissolves readily in cold water, leaving a trace of insoluble residue; a 0·4 % solution was subjected to various qualitative tests. Acid $KMnO_4$ solution was reduced in the cold. Positive biuret and ninhydrin reactions showed the presence of peptide linkages and amino-groups respectively, and that these were due to the presence of tyrosine residues was indicated by positive xanthoproteic and Millon's reactions. The presence of arginine was indicated by the Sakaguchi reaction, but the aldehyde test for tryptophan gave negative results. An immediate bulky precipitate was obtained, with phosphotungstic acid in 5 % H_2SO_4 and a precipitate on standing with neutral lead acetate, but no precipitate was obtained with 10 % trichloroacetic acid or tannic acid solution. A positive Molisch's reaction was obtained, but no furfuraldehyde was obtained on heating a sample with 12 % HCl, and Foulger's test for ketoses was also negative.

Whilst the alcohol-soluble fraction may be expected to contain any low-molecular substances liberated by acetic acid hydrolysis, e.g. sugars, amino-acids, etc., and the above quantitative and qualitative examinations would appear to confirm the presence of small amounts of these substances, there remains nevertheless about 10 % of N unaccounted for by amino-N. The presence of arginine and tyrosine and the precipitability of the fraction with phosphotungstic acid and neutral lead acetate are not inconsistent with the view that a water-soluble polypeptide may be present. Attempts to break down the substance by tryptic digestion and hydrolysis with mineral acids to produce free amino-acids have so far been unsuccessful.

Action of trypsin on alcohol-soluble fraction. 0·15 g. of the dried substance was dissolved in 15 ml. of a veronal-HCl buffer (*p*H 8·5), 0·003 g. trypsin (Fairchild Bros. and Foster, New York) added and the mixture incubated at 37° and *p*H 8·2–8·5; a further 0·003 g. trypsin was added after 2 days' digestion. Before addition of trypsin 7·5 % of the total N was present as amino-N (Van Slyke); this increased to 9·7 % after 2 days' digestion and 11·3 % after 5 days' digestion. It is evident, therefore, that the substance was not degraded to any significant extent by trypsin.

Acid hydrolysis of alcohol-soluble fraction. Acid hydrolysis of the alcohol-soluble fraction was investigated under the same conditions as those employed for the hydrolysis of the insoluble polypeptide fraction. 10 mg. samples of the dried solid were heated at 100° in sealed tubes with 1 ml. quantities of 10*N* formic acid containing 3·6 % HCl. After 2 hr. heating 12·4 % of the total N was liberated as amino-groups and 16·4 % after 7 hr., as compared with 45 % after 7 hr. in the case of the water-insoluble polypeptide. The action of conc. HCl [cf. Mitchell & Hamilton, 1929] on the alcohol-soluble fraction was also investigated. 10 mg. quantities of the substance were heated at 100° in sealed tubes with 1 ml. concentrated HCl; after 6 hr. 18·0 % of the total N was liberated as amino-N and 18·3 % after 8 hr. heating. It appears unlikely that the alcohol-soluble component of the antigen is a polypeptide and the nature of this fraction remains obscure; investigation of this problem is being continued.

The lipid components of the antigenic complex

Taken as a whole the products of the acetic acid hydrolysis of *Bact. typhosum* Ty2 antigenic complex, which are soluble in organic solvents (light petroleum, ether, ethyl alcohol and acetone) constitute a total of only 2·7 % of the original antigen (Table 1). (In another experiment under similar conditions 4·3 % of substance soluble in these solvents was obtained.) Although other workers have isolated much larger amounts of lipid components of bacterial antigens [Morgan & Partridge, 1940; Soru & Combiesco, 1940] our *Bact. typhosum* Ty2 antigen does not appear to liberate a significant quantity of lipid components on acetic acid hydrolysis.

The light petroleum- and ether-soluble fractions described in Table 1 were combined by dissolving in an ether-alcohol mixture and N and P determinations made, giving N 0·6 %, P 0·5 % and N/P ratio 2·6. 0·15 g. of the lipid fraction was dissolved in 20 ml. ether and extracted with aqueous Na_2CO_3, when the bulk of the lipid material passed into the alkaline aqueous solution. This latter fraction was recovered by acidification and extraction with ether. The ethereal solution was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness, yielding 0·094 g. of a partially crystalline yellow-orange fatty substance. On analysis it gave N 1·2 %, P 1·0 %, N/P ratio 2·8. The material unextracted from ethereal solution by aqueous Na_2CO_3 was also recovered and found to be almost completely soluble in acetone. It appears, therefore, from a preliminary examination, that the lipid fraction consists predominantly of a mixture of fatty acids while a phospholipin may also be present in small amounts.

The action of trypsin on the tryptic digest antigen of Bact. typhosum *Ty2*

The antigens of Gram-negative organisms have generally been regarded as resistant to digestion by proteolytic enzymes [Boivin & Mesrobeanu, 1938, 1]. Morgan & Partridge [1940] have, however, been able to destroy the antigenicity of the *B. dysenteriae* (Shiga) antigen by prolonged treatment with relatively large quantities of trypsin. By tryptic digestion they degraded the complete antigen (a phospholipin-polysaccharide-polypeptide complex) into an antigenically inactive phospholipin-polysaccharide complex. It seemed unlikely that the *Bact. typhosum* Ty2 antigenic complex, which is isolated in an immunologically active and toxic condition by prolonged tryptic digestion of the bacterial cells, would be susceptible to attack by trypsin, but the further effect of relatively large quantities of the enzyme for a long period was investigated as follows. 0·206 g. of tryptic digest antigen was dissolved in 20 ml. veronal-HCl buffer (*p*H 8·5), and the solution treated with 0·008 g. trypsin (Fairchild Bros. and Foster, New York); the digest was incubated at 37° and *p*H 8·2–8·5. The course of the tryptic digestion was determined on 1 ml. samples of digest by the Van Slyke method for determination of amino-N. After 60 hr. digestion a further 0·03 g. trypsin was added and the experiment continued for 12 days in all. The results are summarized in Table 5.

Thus even when the antigen is submitted to prolonged attack by relatively large amounts of trypsin (approximately 20 times as much as that used in the tryptic digestion of the bacterial bodies) it undergoes an insignificant degradation

Table 5. *Rate of liberation of amino-N during tryptic digestion of* Bact. typhosum *Ty2 antigenic complex*

Time in hr.	Amino-N per ml. (mg.)	Total N per ml. (mg.)	Amino-N as % of total N
0	0·068	0·747	9·0
3	0·059	0·747	8·0
60	0·082	0·747	11·0
72	0·099	0·747	13·3
288	0·108	0·747	13·5

as indicated by liberation of only a further 4·5 % of the N as amino-groups. It is concluded that the tryptic digest antigen of *Bact. typhosum* Ty2 is stable towards further attack by this enzyme.

DISCUSSION

Two main constituents of the antigenic complex of *Bact. typhosum* Ty2 have been isolated and investigated. These consist of a polysaccharide, which constitutes 50–60 % of the antigen and an acidic, water-insoluble polypeptide, constituting about 16 % of the complex. The polysaccharide, which has been obtained free from N and all but traces of P, gives on analysis C, 44·4; H, 7·1; acetyl, 3 %: and has been shown to consist of *d*-mannose, *d*-galactose and *d*-glucose units. An identical polysaccharide, $[\alpha]_D^{20^\circ} + 114^\circ$, $[\alpha]_{5461}^{20^\circ} + 140^\circ$, R.V. = 83 % apparent glucose, has been isolated directly from the dried bacterial cells by extraction with dilute acetic acid. Precipitation reactions with 'pure' O- and Vi-antisera have shown that the polysaccharide reacts specifically with O-antiserum but not with Vi-antiserum (in contrast to the antigenic complex from which it is derived), and is to be regarded as the O-specific hapten of *Bact. typhosum*. The polysaccharide probably represents, in a pure form, the soluble specific polysaccharide of *Bact. typhosum*, which White [1929] obtained, together with similar polysaccharides from other Salmonellas. Topley *et al.* [1937] also reported 'a highly dextrorotatory polysaccharide' on hydrolysis of virulent strains of *Bact. typhosum*. Earlier studies on crude bacterial polysaccharides have indicated that, as compared with the complete antigenic complex, they are relatively non-toxic and lacking in antigenic properties. Soru & Combiesco [1940] have isolated a polysaccharide on hydrolysis of the O-antigen of *Bact. typhosum*, with dilute acetic acid, which is probably identical with our polysaccharide though not yet pure. The polysaccharide of Soru & Combiesco is free from N, gives 72·4 % of reducing sugars on hydrolysis, is highly dextrorotatory ($[\alpha] + 125^\circ$) and contains 11·65 % of ash and 2·88 % of P (results, except that for P, calculated on an ash-free basis).

The polysaccharide hapten, which determines the specificity of the O-antigen of *Bact. typhosum*, has a parallel in the polysaccharide haptens of other organisms such as *B. dysenteriae* (Shiga), the specific polysaccharide of which, prepared by similar methods to those used in the present work, contains N (1·6 %), gives 97 % of reducing sugars on hydrolysis and is dextrorotatory ($[\alpha]_{5461} = +98^\circ$). This polysaccharide is built up, in part, of *d*-galactose, *l*-rhamnose and *N*-acetylglucosamine units [Morgan, 1938; Jolles & Morgan, 1940].

The other well-characterized component of the *Bact. typhosum* Ty2 antigenic complex, the polypeptide-like component, probably plays a non-specific role in the complex antigen and is to be regarded as producing, in combination with the polysaccharide, a molecule of sufficient complexity to possess antigenic properties, the specific nature of which is dominated by the polysaccharide. The polypeptide component contains 8·2 % N and 2·4 % P; tyrosine and arginine have been identified. The compound is fairly readily hydrolysed with 10N formic acid which contains 3·6 % HCl at 100° with liberation of 48 % of the N as free amino-groups (Van Slyke), but it is relatively very resistant to tryptic digestion. Soru & Combiesco [1940] obtained 21·7 % of an ether- and water-insoluble fraction from *Bact. typhosum* O-antigen. This fraction contained 6·7 % of N and only a trace of P. On hydrolysis with 2·5 % HCl the polypeptide-like fraction of Soru & Combiesco liberates a further ether-soluble constituent and virtually the whole of the N of the substance is liberated as amino-N. The latter observation appears to us surprising in view of the presence in the compound of arginine, and probably other amino-acids containing N other than as amino-groups. The corresponding polypeptide of *B. dysenteriae* (Shiga) contains 11·6 % N, tyrosine and arginine are present and again about 50 % of the total N is liberated as amino-N on acid hydrolysis [Morgan & Partridge, 1940].

Two other components of the antigenic complex have also been isolated but not yet investigated in any detail. Mixed lipid fractions amounting to 3–4 % were isolated in several experiments and appear to consist of a mixture of fatty acids together with a phospholipin. Soru & Combiesco [1940] obtained 15·8 % of an ether-soluble fraction from the *Bact. typhosum* O-antigen on hydrolysis with dilute acetic acid. The fraction was shown to consist of a mixture of fatty acids. Even larger amounts of lipid fractions have been isolated by Boivin & Mesrobeanu [1938, 2] on hydrolysis, with strong HCl, of the isolated O- and Vi-antigens of *Bact. typhosum*, which liberated 27·2 and 26·5 % of 'acides gras', respectively.

An alcohol-soluble nitrogenous fraction which comprises 10–20 % of the antigen has also been obtained. It contains 13·4 % N, the bulk of which is not present as free amino-groups and the percentage of these does not increase markedly on treatment with trypsin or on hydrolysis with acids. The nature of this component is still undetermined, and this problem is under investigation. The parts played by the alcohol-soluble component and the lipids in the complex antigen cannot yet be postulated but it is thought that they are of less fundamental importance than the polysaccharide and polypeptide components. Morgan & Partridge [1940] have shown that, although the *B. dysenteriae* (Shiga) antigen exists in the bacterial cell as a phospholipin-polysaccharide-polypeptide complex, the phospholipin component may be removed leaving a polysaccharide-polypeptide complex which is still specifically antigenic.

Preliminary work on an O-antigenic strain of *Bact. typhi-murium* has yielded similar results to those obtained with *Bact. typhosum* Ty2, and led to the isolation of a polysaccharide fraction, an insoluble polypeptide-like component, a small amount of a lipid substance and a soluble nitrogenous component.

SUMMARY

1. The *Bact. typhosum* Ty2 antigen has been shown to consist of the following constituents, into which it may be dissociated on gentle hydrolysis: a polysaccharide component (50–60 %), an insoluble polypeptide (about 16 %), a soluble nitrogenous component (10–20 %) and a small lipid component (3–4 %).

2. The polysaccharide represents the O-specific hapten of the antigen; it has a reducing value on hydrolysis of 83 % in terms of glucose and is highly dextrorotatory ($[\alpha]_D^{20^\circ} = +114^\circ$). It appears to be free from ketose, pentose and uronic acid residues but yields *d*-galactose, *d*-mannose and *d*-glucose on hydrolysis. An identical polysaccharide has been prepared directly from the dried organisms by extraction with dilute acetic acid.

3. The polypeptide component is insoluble in water and soluble in dilute alkalis. It contains 8·2 % N, partly in the form of tyrosine and arginine units and about 50 % of the total N is liberated as amino-groups on acid hydrolysis.
4. The nature of the soluble nitrogenous component is, as yet, undetermined.
5. The antigen of *Bact. typhosum* Ty2 is resistant to tryptic digestion.

These studies and others in the same general field are of necessity extremely expensive. They have involved the growth of relatively enormous masses of pathogenic bacteria and laborious and large-scale chemical manipulations. They have been rendered possible by grants received from many different sources: from the Leverhulme Trust, the Pilgrim Trust, Imperial Chemical Industries, Ltd., and from a fund raised by the late Sir Austen Chamberlain, to which many generous private donors have contributed. One of us (G.G.F.) has held a Grocers' Company Scholarship for Medical Research. To all these we would express our thanks.

The authors wish to thank Prof. H. Raistrick for much valuable advice and criticism.

REFERENCES

Boivin & Mesrobeanu (1934). *C.R. Soc. Biol., Paris*, **115**, 304, 309.
—— —— (1937). *C.R. Soc. Biol., Paris*, **124**, 442.
—— —— (1938, 1). *Ann. Inst. Pasteur*, **61**, 426.
—— —— (1938, 2). *C.R. Soc. Biol., Paris*, **128**, 5.
—— —— & Mesrobeanu (1933). *C.R. Soc. Biol., Paris*, **114**, 307.
—— —— —— (1935). *Arch. roum. Path. exp. Microbiol.* **8**, 45.
Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
Freeman, Challinor & Wilson (1940). *Biochem. J.* **34**, 307.
Jolles & Morgan (1940). *Biochem. J.* **34**, 1183.
Malek (1939). *C.R. Soc. Biol., Paris*, **132**, 15.
Meyer (1938). *C.R. Soc. Biol., Paris*, **129**, 485.
Miles & Pirie (1939, 1). *Brit. J. exp. Path.* **20**, 83.
—— —— (1939, 2). *Brit. J. exp. Path.* **20**, 109.
—— —— (1939, 3). *Brit. J. exp. Path.* **20**, 278.
Mitchell & Hamilton (1929). The Biochemistry of the Amino Acids. New York.
Morgan (1937). *Biochem. J.* **31**, 2003.
—— (1938). *Helv. chim. Acta*, **21**, 469.
—— & Partridge (1940). *Biochem. J.* **34**, 169.
Raistrick & Topley (1934). *Brit. J. exp. Path.* **15**, 113.
Stewart & Hendry (1935). *Biochem. J.* **29**, 1683.
Soru & Combiesco (1940). *C.R. Soc. Biol., Paris*, **133**, 498.
Topley, Raistrick, Wilson, Stacey, Challinor & Clark (1937). *Lancet*, **1**, 252.
White (1929). *J. Path. Bact.* **32**, 85.

60. THE 3:5-DINITROBENZOYL DERIVATIVES OF THE AMINO-ACIDS AND THEIR USE IN SEPARATING THE ISOMERS OF LEUCINE AND VALINE

By BERNARD WILLIAM TOWN

From the Department of Chemistry, The Medical College of St Bartholomew's Hospital, London

(*Received 26 March 1941*)

In recent papers, Saunders [1934; 1938] has described the preparation of the 3:5-dinitrobenzoyl derivatives of certain amino-acids and has suggested their use for purposes of identification. This possibility has been confirmed by the present author; however, certain discrepancies have been observed between published data and present experience and these observations are embodied in this communication.

In the first place, the reaction between 3:5-dinitrobenzoyl chloride and amino-acids has been found to be much slower than stated by Saunders. Reaction times of the order of 30 min. have been found, compared with quoted times of 2 min. for similar quantities of material. It was also observed that some 3:5-dinitrobenzoic acid is always obtained during benzoylation, even when molecular proportions of reactants are employed, and this can be very troublesome. It is suggested by Saunders that practically no 3:5-dinitrobenzoic acid is formed during the reaction.

It has also been found that the melting points of some of the derivatives differ considerably from those quoted by Saunders, and such differences are noted in the experimental section of the paper. Also in the cases of valine and leucine more than one derivative has been isolated. The melting points of the derivatives which the author has been able to prepare are quoted in Table 1. These melting points have been consistently obtained in this laboratory and are believed to be correct.

Table 1. *3:5-Dinitrobenzoyl derivatives of amino-acids*

Derivative of:	M.P. °C
Glycine	182·2, shrinks 181·4
l(+)-Alanine	177, softens 176·6
l(−)-Threonine	181, softens 180
l(−)-Serine	183, shrinks 112
l(−)-Cystine	Derivative not suitable
dl-Valine	211·4, softens 210·8
l(+)-Valine	207
*nor*Valine (?)	182
l(−)-Leucine	188, softens 187
l(+)-*iso*Leucine	170·4, softens 169·2
*nor*Leucine (?)	163
dl-Phenylalanine	161
l(−)-Tyrosine	No derivative obtainable
l(−)-Aspartic acid	184·8, softens 184·4
Na-salt of aspartic acid derivative	218
l(+) *d*(−) -Glutamic acid *dl*	104
r-Glutamic acid	204
Synthetic *β*-hydroxyglutamic acid	229·5, darkens 228
3:5-Dinitrobenzoic acid	207·8
3:5-Dinitrobenzoyl chloride	74

A derivative has been isolated from aspartic acid, whereas Saunders isolated no derivative, but deduced from a formaldehyde titration that 50 % of the aspartic acid remained unchanged. Experience has shown that this derivative is very easily hydrolysed and may be decomposed if it is kept in a solution more acid than *p*H 2 for any length of time.

The crystalline forms of these derivatives are described in the experimental section, and photographs of most of them have been taken to ensure a more reliable record.

As many of the derivatives have similar melting points, determination of melting point alone is not sufficient to characterize them. Their melting points are, however, very sensitive to the presence of impurities, particularly 3:5-dinitrobenzoic acid, and it is convenient to prepare standard preparations of all these derivatives so that mixed melting points can be taken.

A very important observation has been made in that the various isomeric valines and leucines can be separated by the fractional precipitation of their 3:5-dinitrobenzoyl derivatives at different *p*H. No such separation could be effected by fractional crystallization. The great value of this separation lies in the fact that no means of estimating the proportion of the different isomers of valine and leucine in a mixture has hitherto been available. It was during the course of such separation that derivatives were isolated from valine and leucine which differed from the normal in melting point, but were yet pure. It is suggested that they are the derivatives of *nor*valine and *nor*leucine. This is subject to confirmation. They are indicated by a query in Table 1.

It has been found very difficult to free any derivative from traces of 3:5-dinitrobenzoic acid once it has been contaminated. In every case, therefore, precipitation of the 3:5-dinitrobenzoyl derivative has been effected gradually by adding standard acid to the alkaline solution in stages, filtering off each precipitate as it forms, and recording the *p*H by means of indicators. The derivatives of dicarboxylic amino-acids are insoluble in benzene; this enables any free 3:5-dinitrobenzoic acid to be separated by continuous extraction in a Jena sintered glass apparatus as used for rubber analysis. This treatment was not suitable for the derivatives of monobasic monoamino-acids.

EXPERIMENTAL

3:5-Dinitrobenzoyl chloride hydrolyses very rapidly on exposure to air. It is best to obtain a freshly crystallized specimen and put it up in 1 g. portions in dry tubes immediately; such a specimen has been kept in good condition for more than a year. When pure it crystallizes in needles and melts at 74°.

3:5-Dinitrobenzoic acid which is always obtained as a by-product crystallizes in thick rectangular plates, M.P. 207·8°.

In all cases described below 1 g. of 3:5-dinitrobenzoyl chloride was used, and was added slowly, with mechanical shaking between each addition, to the calculated amount of amino-acid in 5 ml. water, containing the calculated quantity of 2 *N* NaOH (2 mol.). Excess of acid chloride was not used owing to the difficulty of separating free 3:5-dinitrobenzoic acid from the amino-acid derivative.

Derivatives of individual amino-acids

Glycine. On acidifying the reaction mixture with *N* HCl to *p*H 3·3 a fine crystalline precipitate was obtained, M.P. 158–160°. Crude yield 1·06 g. (91 % of theory). It was found difficult to separate from traces of 3:5-acid by crystallization, but after crystallizing twice from 10 % acetic acid and once from water, it gave long fine needles, M.P. 182·2°, shrinking at 181·4°.

Loss in wt. on drying at 110° = 6·5 % (theory for $C_9H_7O_7N_3, H_2O$, 6·27 %). Saunders [1934] quotes M.P. 179°.

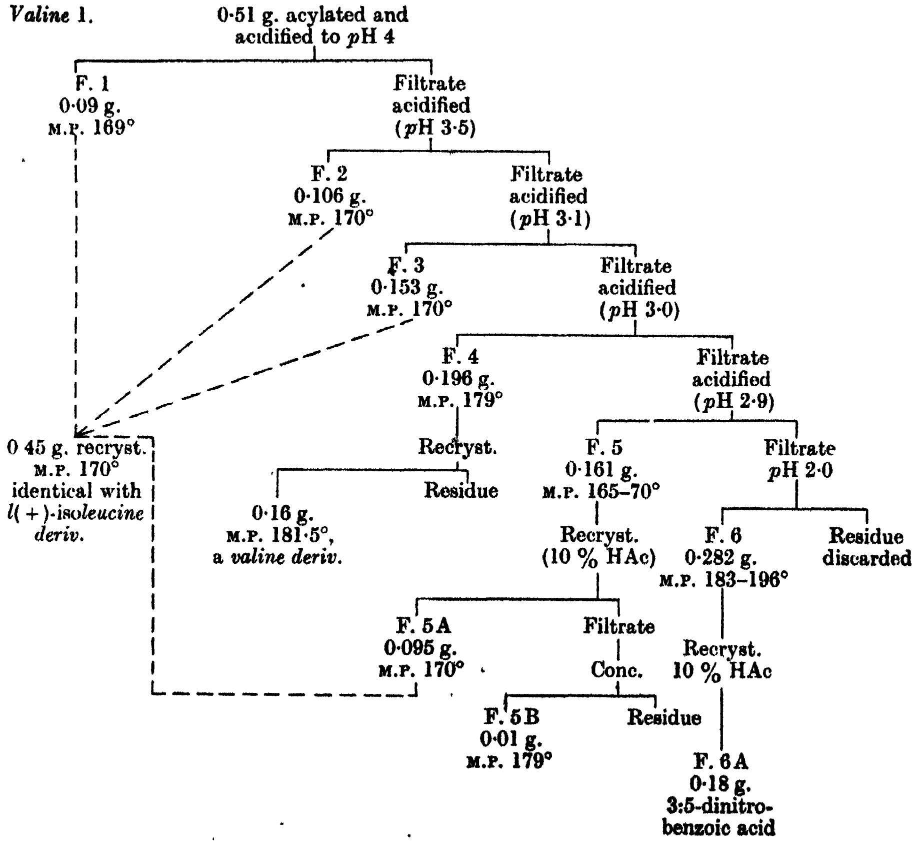
l(+)- *and* dl-*alanine.* On acidifying the solution after benzoylation of *dl*-alanine (B.D.H.) to *p*H 2·6, and leaving in the refrigerator overnight, 0·729 g. of derivative, M.P. 176°, softening at 172° was obtained (63 % yield). After crystallization from 10 ml. 10 % acetic acid it yielded 0·61 g., M.P. 177° sharp, containing no water of crystallization.

A sample of *l*(+)-alanine also gave a derivative with the same melting point (58 % yield); mixed M.P. with the *dl*-derivative, 174°, softening at 172°. The above data agree with those quoted in the literature.

Valine. Great discrepancies have been observed here between the figures quoted in the literature and those actually obtained. The derivative has been prepared from three samples of valine, first from a specimen of *dl*-valine, and then from two samples of *l*(+)-valine which had been presented to the author.

dl-*Valine* (B.D.H.) gave, on bringing the reaction mixture to *p*H 2·8, a crude product M.P. 184° (68·5 % yield). This, after purification, crystallized in glistening flat rectangular platelets, M.P. 211·4°, softening at 210·8°. (Found: C, 46·45; H, 4·27; N, 13·42 %. $C_{12}H_{13}O_7N_3$ requires C, 46·35; H, 4·18; N, 13·50 %.) This derivative contains no water of crystallization, whereas Saunders found for the derivative of *l*(+)-valine M.P. 157–158°, and also 1 mol. of water of crystallization.

l(+)-*Valine.* As both samples of natural valine proved to be mixtures and as the results differed with each sample, it will be easier to record the findings diagrammatically.



In all, taking into account various minor fractions, there were isolated: 0·508 g. *l*(+)-*iso*leucine derivative, M.P. 170·2°, equivalent to 0·205 g. *l*(+)-*iso*leucine or 40 % of the initial material; 0·196 g. valine derivative, M.P. 181·5°, equivalent to 0·074 g. of valine or 14·5 % of the starting material.

As the total yield of derivatives (54·5 %) is of the same order as the yield normally obtained in an acylation with 3:5-dinitrobenzoyl chloride, it seems reasonable to suppose that this sample of so-called valine was in reality a mixture of *iso*leucine and valine in the proportion of approximately 74 % *iso*leucine and 26 % valine.

It is important to note that the M.P. of this valine derivative is far removed from that obtained with *dl*-valine (B.D.H.) and also from that obtained by Saunders [1934] who gives M.P. 157–158°.

Analysis of these two derivatives by Schoeller gave the following results: *iso*leucine derivative: found: C, 47·87; H, 4·40; N, 13·04 %; calc. C, 48·00; H, 4·62; N, 12·92 %; valine derivative: found: C, 46·57; H, 4·36 %, N, 13·35 %; calc. C, 46·35; H, 4·36; N, 13·50 %.

Valine 2. From this sample there have been isolated (see diagram): 0·31 g. of *iso*leucine derivative, M.P. 170°, equivalent to 0·115 g. of free *iso*leucine or 7·5 % of the initial weight of substance; 0·64 g. of a valine derivative, M.P. 182°, equivalent to 0·26 g. of free valine, or 17 % of the starting material (this derivative is identical with that isolated from valine 1); 1·22 g. of another valine derivative, M.P. 207°, equivalent to 0·458 g. of free valine or 30 % of the starting material; this valine derivative is apparently identical with that obtained from synthetic *dl*-valine (B.D.H.) as no lowering of M.P. occurs on mixing. As this synthetic valine is certainly that variety with a *gem*-dimethyl group, it is possible to fix the structure of the valine giving the 3:5-dinitrobenzoyl derivative, M.P. 207°. The structure of the valine giving the 3:5-dinitrobenzoyl derivative, M.P. 182°, remains to be determined. It is suggested provisionally that this is *nor*valine.

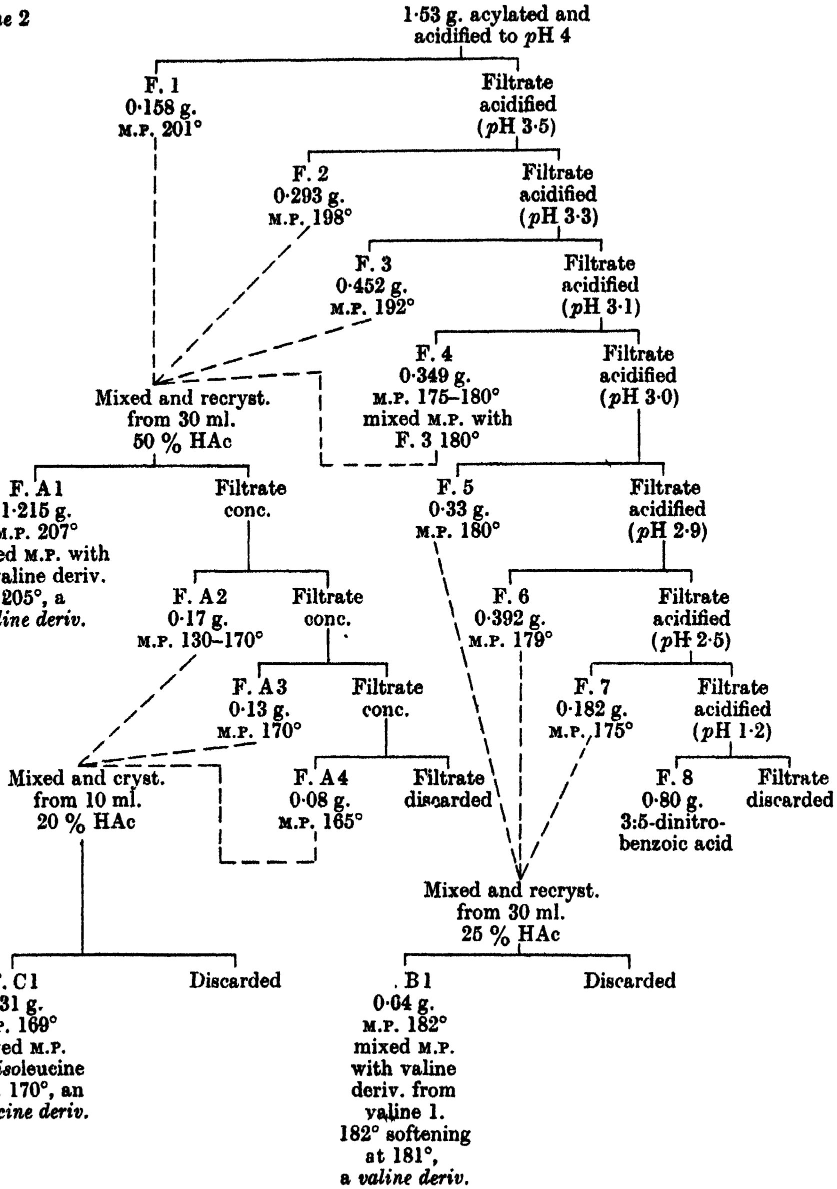
The three derivatives isolated from valine 2 were analysed by Schoeller: 3:5-dinitrobenzoyl derivative, M.P. 182°: C, 46·52; H, 4·39; N, 13·31 %; 3:5-dinitrobenzoyl derivative, M.P. 207°: C, 46·48; H, 4·23; N, 13·50; valine derivative requires C, 46·35; H, 4·18; N, 13·50 %; 3:5-dinitrobenzoyl derivative, M.P. 170°: C, 47·78; H, 4·42; N, 13·02 %; *iso*leucine derivative requires C, 48·00; H, 4·62; N, 12·92 %. The 3:5-dinitrobenzoyl derivative, M.P. 182°, crystallizes in very long but narrow rectangular rods, while the derivative with M.P. 207° crystallizes in large thin hexagonal plates which often have an irregular outline The derivative from *dl*-valine, M.P. 211·4°, crystallizes in the same form as the derivative with M.P. 207°, which confirms their identity. Photographs have been taken of these crystals.

It is interesting to compare the yields of 3:5-dinitrobenzoyl derivatives from these two samples of valine:

	3:5-Dinitrobenzoyl derivatives isolated			
	M.P. 207° %	M.P. 182° %	M.P. 170° %	Total %
Valine 1	0	14·5	40	54·5
Valine 2	30	17	7·5	54·5

The yield in either case is 54·5 % expressed as amino-acid isolated in terms of starting material. This yield is of the same order as is usually obtained in acylating pure amino-acids, so that if no other amino-acids are assumed to be

Valine 2


1·53 g. acylated and acidified to pH 4
F. 1 0·158 g. M.P. 201°
Filtrate acidified (pH 3·5)
F. 2 0·293 g. M.P. 198°
Filtrate acidified (pH 3·3)
F. 3 0·452 g. M.P. 192°
Filtrate acidified (pH 3·1)
F. 4 0·349 g. M.P. 175–180° mixed M.P. with F. 3 180°
Filtrate acidified (pH 3·0)
Mixed and recryst. from 30 ml. 50 % HAc
F. A1 1·215 g. M.P. 207° mixed M.P. with dl-valine deriv. 205°, a valine deriv.
Filtrate conc.
F. 5 0·33 g. M.P. 180°
Filtrate acidified (pH 2·9)
F. A2 0·17 g. M.P. 130–170°
Filtrate conc.
F. 6 0·392 g. M.P. 179°
Filtrate acidified (pH 2·5)
F. A3 0·13 g. M.P. 170°
Filtrate conc.
F. 7 0·182 g. M.P. 175°
Filtrate acidified (pH 1·2)
Mixed and cryst. from 10 ml. 20 % HAc
F. A4 0·08 g. M.P. 165°
Filtrate discarded
F. 8 0·80 g. 3:5-dinitrobenzoic acid
Filtrate discarded
Mixed and recryst. from 30 ml. 25 % HAc
F. C1 0·31 g. M.P. 169° mixed M.P. with isoleucine deriv. 170°, an isoleucine deriv.
Discarded
B1 0·04 g. M.P. 182° mixed M.P. with valine deriv. from valine 1. 182° softening at 181°, a valine deriv.
Discarded

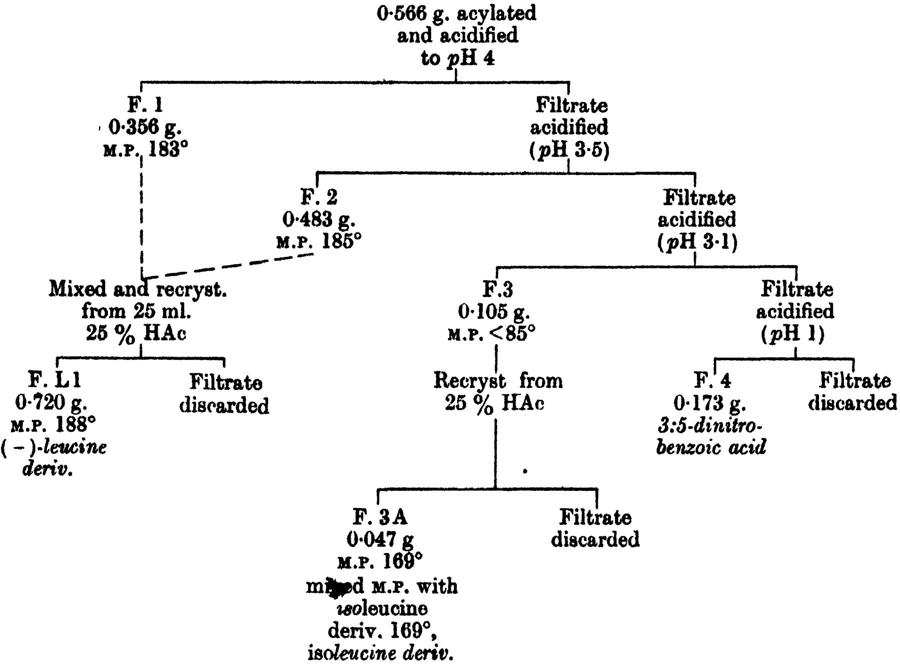
present, it is possible to calculate the original percentage composition of the mixtures:

	Valine 1 %	Valine 2 %
Valine	Nil	55
*nor*Valine (?)	26·6	31·2
*iso*Leucine	73·4	13·8
	100·0	100·0

While these figures are unlikely to be accurate to more than 1–2 %, they do give some idea of the compositions of the two samples and indicate that neither was pure. Until methods for the isolation of amino-acids have been perfected it would seem idle to give tables showing the percentage composition of proteins.

The melting point quoted by Saunders for the valine derivative is 157–158°, and he states that it crystallizes with 1 H_2O. The two derivatives isolated in the present work are anhydrous, and neither melting point is in the vicinity of that quoted.

l(−)-*Leucine*. For this preparation a sample of *l*(−)-leucine was obtained from Schuchardt's, and was of guaranteed purity.



This *l*(−)-leucine derivative had m.p. 188°, softening at 187·2°, on a standard thermometer; it contained no water of crystallization. These data agree with those quoted by Saunders.

The yield obtained was 61 % of theory; there was also obtained 3·4 % of *l*(+)-*iso*leucine derivative, m.p. 169°. Thus this amino-acid, in spite of its label, was not pure and consisted of a mixture of 95 % *l*(−)-leucine and 5 % *l*(+)-*iso*leucine. A sample of leucine isolated from the hydrolysis of horsehair was also used to prepare the derivative as this was a specimen thought to be of great purity.

From 1·132 g. of this there were isolated 1·32 g. leucine derivative, m.p. 188°, equivalent to 0·532 g. leucine or 47·2 % of the original material. This derivative crystallizes in very thin rectangular plates with one or both ends cut off obliquely. There was also isolated another derivative, m.p. 163°, also crystallizing in very thin rectangular plates, but with all ends perfectly square, in amount corresponding to 0·097 g. leucine or 8·55 % of the starting material. (Found: C, 47·75; H, 4·60; N, 13·02 %. Theory: C, 48·00; H, 4·61; N, 12·91 %.)

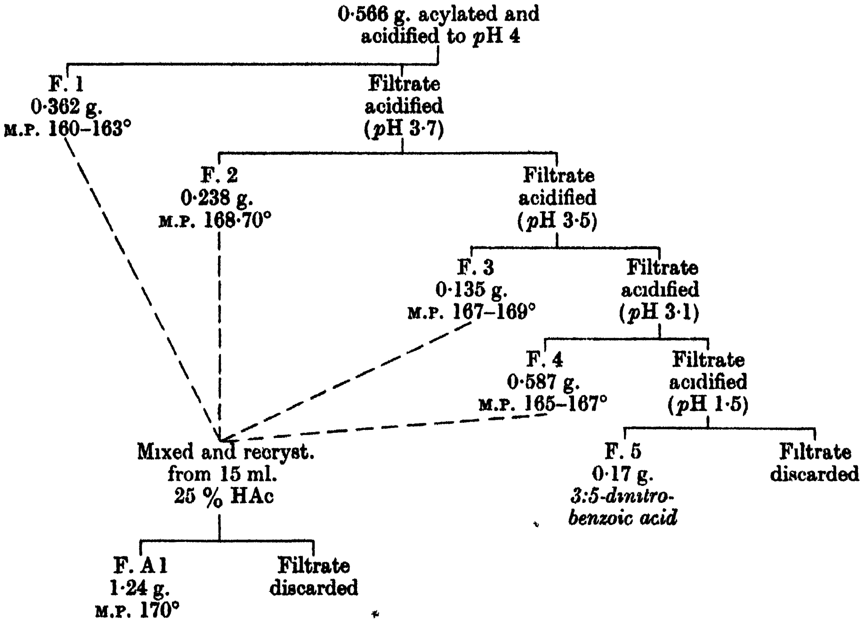
The total yield of leucine derivatives from this specimen of leucine is thus 55·7 % of the initial leucine which is of the same order as is usually obtained.

One may calculate on this basis that the composition of this sample of leucine was approximately:

	%
l(−)-Leucine	84·8
*nor*Leucine (?)	15·2
	100·0

It is assumed provisionally that this derivative, which analyses for a leucine derivative but does not correspond with that either of leucine or *iso*leucine, is that of *nor*leucine.

l(+)-iso*Leucine*. A sample of pure *l*(+)-*iso*leucine from Schuchardt was used for the preparation of this derivative.


0·566 g. acylated and acidified to *p*H 4
F. 1
0·362 g.
M.P. 160–163°
Filtrate acidified (*p*H 3·7)
F. 2
0·238 g.
M.P. 168·70°
Filtrate acidified (*p*H 3·5)
F. 3
0·135 g.
M.P. 167–169°
Filtrate acidified (*p*H 3·1)
F. 4
0·587 g.
M.P. 165–167°
Filtrate acidified (*p*H 1·5)
F. 5
0·17 g.
3:5-dinitro-benzoic acid
Filtrate discarded
Mixed and recryst. from 15 ml. 25 % HAc
F. A1
1·24 g.
M.P. 170°
Filtrate discarded

This derivative crystallizes in long narrow rectangular plates. A derivative was also prepared from the *iso*leucine isolated from gliadin by Town [1929], and this also had M.P. 170°.

Serine. A sample of pure serine isolated by the author from gliadin was used in this preparation. 0·457 g. serine was acylated, but as the derivative did not precipitate until the solution was acidified to *p*H 1, it was contaminated with free 3:5-dinitrobenzoic acid which was difficult to remove by crystallization from 25 % acetic acid (10 ml.). The weight of crude derivative was 0·964 g. and its M.P. 157°, softening at 140°. It was extracted continuously with benzene in a Jena sintered glass extraction thimble for 45 min. The benzene on evaporation yielded 0·32 g. 3:5-dinitrobenzoic acid. The residue insoluble in benzene, 0·61 g., M.P. 183°, was crystallized from water and then had M.P. 183°, softening at 181° and shrinking at 112°, crystallizing in needles. Yield 46·8 %. This serine derivative was dried at 110°: loss in weight, 5·45 %; calc. for $C_{10}H_9N_3O_8$, H_2O, 5·66 %. (Found: C, 37·99; H, 3·77; N, 13·22 %; calc. for hydrated serine derivative: C, 37·90; H, 3·37; N, 13·24 %. The anhydrous derivative has M.P. 183°. Saunders quotes the melting point of serine derivative as 94–95°.

l(−)-*Threonine.* A pure sample of this amino-acid isolated by the author from gliadin was used for the preparation of the derivative. The same difficulty was encountered as in the preparation of the serine derivative, that the solution had to be acidified to *p*H 2 before any precipitate was formed. On leaving in the ice-chest for some hours a precipitate of 0·07 g. 3:5-dinitrobenzoic acid formed. The solution had now to be left for over a week.

117 mg. of threonine were benzoylated, and 79 mg. of a derivative, M.P. 135°, slowly separated. It was extracted continuously with benzene and gave 34 mg. residue, which after crystallizing from 5 ml. 10 % acetic acid had M.P. 181°, softening at 180°. It crystallized in long thin rectangular rods. Yield 11·2 %. (Found: C, 41·83; H, 3·60; N, 13·30 %; calc. for $C_{11}H_{11}N_3O_8$: C, 42·18; H, 3·51; N, 13·42 %.)

The yields of derivatives of both these hydroxy-acids are low, presumably owing to their greater solubilities.

Cystine. The derivative is not really suitable for the identification of cystine, and although the author's experience differs rather from the description of Saunders, its description is unwarranted.

Phenylalanine. dl-Phenylalanine (B.D.H.), 0·715 g., was acylated, and after bringing to *p*H 7, an oil was precipitated. Sufficient methyl alcohol was added just to dissolve the oil and the solution was left for 24 hr.; 0·90 g. crystals separated, M.P. 147°, softening at 142°. The filtrate was brought to *p*H 4 and treated in the same way; a second crop was obtained, 0·22 g., M.P. 110°, softening at 105°. Only 3:5-dinitrobenzoic acid was obtained on further acidification.

The mixed crops were crystallized from 30 ml. 60 % methyl alcohol and gave 0·78 g., M.P. 160°, softening at 107°. Yield 50·3 %. On recrystallization from 25 ml. 50 % acetic acid, the derivative had M.P. 161°, softening at 160°, and crystallized in thin rectangular rods: wt. 0·57 g. The substance has no water of crystallization. (Found: C, 53·64; H, 3·90; N, 11·60 %; calc. for $C_{16}H_{13}N_3O_7$: C, 53·50; H, 3·62; N, 11·70 %.)

Saunders quotes M.P. 93°, and states that the compound crystallizes with 0·5 mol. H_2O.

Glutamic acid. Natural glutamic acid hydrochloride (0·797 g.) isolated by the author from gliadin was used and, after acylation, the solution was acidified to *p*H 2. A slight oily precipitate separated which rapidly solidified and, after 12 hr. in the ice-chest, 1·073 g. of derivative were obtained, M.P. 130°, softening at 102°. The derivative is quite insoluble in benzene, and after extracting continuously for 1 hr. with boiling benzene the residue (0·570 g.) had M.P. 155°, softening at 153°. The benzene on evaporation gave 0·448 g. of 3:5-dinitrobenzoic acid.

The residue insoluble in benzene was crystallized from 8 ml. 10 % acetic acid and yielded 0·46 g. of a derivative, M.P. 104°, softening at 102°, crystallizing in very long fine needles. It has a water content of 5·47, 5·45 %; $C_{12}H_{11}N_3O_9, H_2O$ requires 5·04 %. The anhydrous derivative has M.P. 182°, softening at 179°. The yield of derivative is 38·4 % of theory. Saunders quotes M.P. 98–99° and finds that it crystallizes with 0·5 ml. H_2O.

From the mother liquors from the separation of glutamic acid as its hydrochloride from a protein hydrolysate, the author has isolated a glutamic acid which yields a 3:5-dinitrobenzoyl derivative, M.P. 204°, and crystallizes in large but very thin hexagonal plates which tend to overlay one another. It crystallizes in the anhydrous condition. This is believed to be the derivative of *r*-glutamic acid; it is certainly not that of *dl*-glutamic acid which gives the derivative M.P. 104° [Town, 1941].

Aspartic acid. Natural aspartic acid (0·58 g.) was acylated and acidified to *p*H 2, when 0·27 g. of 3:5-dinitrobenzoic acid separated. The filtrate was left for 12 hr. in the ice-chest and 0·36 g. of derivative obtained, M.P. 180°, softening at 177°. This was crystallized from 3 ml. 10 % acetic acid in which it was easily soluble: 0·13 g., M.P. 184·8°, softening at 184·4°, shrinking slightly at 100°. From the mother liquor there was also isolated 0·017 g. of the glutamic acid derivative, M.P. 204°, identical with that isolated from the dicarboxylate fraction of gliadin [Town, 1941]. This has been obtained in small quantity from all the specimens of natural aspartic acid (three) which the author has examined.

The aspartic acid derivative readily crystallizes from water, in which, however, it is rather soluble; it is readily decomposed in solutions more acid than *p*H 2. The substance crystallizes with 1 H_2O: loss in weight at 110°, 5·65 %, calc. for $C_{11}H_9N_3O_9$, H_2O 5·22 %. The anhydrous material has C, 40·1; H, 2·90; N, 12·87 %; calc. for $C_{11}H_9N_3O_9$, C, 40·40; H, 2·75; N, 12·84 %. The yield of derivative is only 25 %, this low figure probably being due to its solubility and to its ready decomposition in acid solution.

In a repetition of this experiment, the solution after benzoylation was only acidified to *p*H 4, when a glistening white precipitate formed consisting of a mass of fine rectangular plates, 0·692 g., M.P. 220°. This was very soluble in water, and originally must have been salted out by the other sodium salts present. It dissolved in 4 ml. H_2O on warming, and separated in shining flat plates on cooling, wt. 0·32 g. (Found: C, corrected for C in ash 37·4; H, 3·09; N, 11·71; Na, 6·66 %; the mono Na salt of 3:5-dinitrobenzoylaspartic acid, $C_{11}H_8N_3O_9Na$, requires C, 37·80; H, 2·30; N, 12·02; Na, 6·60 %.) No other 3:5-dinitrobenzoyl derivative has been found to give a sodium salt which can be salted out in this manner.

β-Hydroxyglutamic acid. A sample of synthetic β-hydroxyglutamic acid (0·232 g.) kindly presented by Prof. Harington was used in this experiment.

After benzoylation, the solution was acidified to *p*H 1 and left for 12 hr. in the ice-chest. The derivative was filtered, wt. 0·426 g., and extracted continuously with benzene. The benzene extract gave 0·30 g. 3:5-dinitrobenzoic acid, and the residue, 0·120 g., M.P. 220–221°, was dissolved in 10 ml. absolute methyl alcohol, filtered from a trace of ferric iron impurity, freed from methyl alcohol and crystallized from 12 ml. 25 % acetic acid. It crystallized in long fine needles, wt. 0·116 g., M.P. darkening at 228° and melting with decomposition at 229·5°. Yield 27 % of theory. (Found: C, 40·39; H, 3·12; N, 11·70 %; calc. for $C_{12}H_{11}N_3O_{10}$, C, 40·40; H, 3·08; N, 11·78 %.)

SUMMARY

It has been confirmed that 3:5-dinitrobenzoyl chloride is a very valuable reagent for the identification of amino-acids. A table of melting points of those derivatives which the author has been able to prepare is given.

The melting points found for some derivatives, notably those of phenylalanine, serine and valine, differ considerably from those quoted by Saunders.

There have been isolated two derivatives from the specimens of valine available, and three derivatives from the samples of leucine at the author's disposal. These are thought to be the derivatives of valine and *nor*valine, and of leucine, *iso*leucine and *nor*leucine respectively. The identity of the *nor*valine and *nor*leucine derivatives is subject to confirmation.

It has been found possible to separate the components in mixtures of the various isomers of valine and leucine by fractional precipitation of their deri-

vatives at differing *p*H. This is important, as no means of separating mixtures of such isomers has hitherto been available.

A derivative has been isolated from aspartic acid, of which the Na salt is readily salted out at *p*H 4. The aspartic acid derivative is easily hydrolysed in solutions more acid than *p*H 2.

No derivative could be isolated from tyrosine.

REFERENCES

Saunders (1934). *Biochem. J.* **28**, 580.
—— (1938). *J. chem. Soc.* p. 1397.
Town (1929). *J. Soc. chem. Ind., Lond.*, **48**, 1248.
—— (1941). *Biochem. J.* **35**, 417.

61. ON THE METHOD OF FORMATION OF PYRUVIC ACID BY BARLEY

By WILLIAM OWEN JAMES, GLADYS MACPHAIL JAMES
AND ARTHUR HUGH BUNTING[1]

From the Department of Botany, Oxford

(*Received 20 March 1941*)

Pyruvic acid is consumed in barley respiration [James & Norval, 1938] and can also be made to accumulate by poisoning the living plant with acetaldehyde or aromatic sulphonic acids [James & James, 1940]. Organic phosphates are present [Arney, 1939] which appear to be connected with respiration in a phosphorylating cycle [James & Arney, 1939]. It is, therefore, a matter of interest to discover whether the sugars consumed in barley respiration pass through such a cycle in their conversion into pyruvic acid.

Material

Leaves of young barley plants (var. Plumage Archer) were cut and frozen overnight at about −12°. They were allowed to thaw at room temperature and their sap was squeezed out through muslin by hand. This sap was usually allowed to stand in the refrigerator (at about 2°) for 24 hr. before use. Any sediment which formed was filtered off, a clear yellow liquid resulting. In a few experiments, as will be described, further purification was attempted. None of these saps by themselves yielded appreciable amounts of pyruvic acid, even after incubation (see p. 589) under the standardized experimental conditions, and they showed very little carboxylase activity. They were, therefore, very convenient for investigating the formation of pyruvic acid from the substances which it was desired to examine. Intact tissues could not be used since they would not have taken up the substrates sufficiently quickly.

Methods

Extraction. After a period of incubation at 30° in the presence of thymol as antiseptic, usually for 48 hr., any pyruvic acid present was separated from the sap. 10 ml. barium acetate were pipetted into the digest and the reaction adjusted to pH 8 by running in $M/5$ baryta. The barium precipitate was centrifuged off and another drop of barium acetate added, to make sure that precipitation was complete. 25 % trichloroacetic acid was then added, to give a final concentration of 5 %. The mixture was allowed to stand for 15 min. and then brought to the boil. The protein flocculum was filtered off and the clear filtrate transferred to a large distillation flask. Distillation was carried out under reduced pressure with a fine stream of air passing through the solution. Water and other volatile substances came over at 50–55° and about 50 mm. pressure.

Identification. Pyruvic acid was identified in the distillate by the isolation of its 2:4-dinitrophenylhydrazone by the method of Peters & Thomson [1934]. The hydrazones obtained were compared with an authentic sample from twice redistilled pyruvic acid, giving M.P. 216°, a canary yellow colour and diamond-shaped platelets, single and clustered. This hydrazone gave a deep red coloration with alcoholic KOH, which was not discharged by adding water.

---

[1] Rhodes Scholar, 1938–41.

The separation of the pure substance from barley preparations presents numerous difficulties, and it was commonly found that the final product showed orange-tinted zones on crystallizing out. To avoid further laborious purifications, well-formed crystals were selected mechanically from the pure yellow zones for determination of the melting-point. Even when the quantity available was fairly considerable, it was found convenient to use the hot-box already described [James & James, 1940], selecting a small number of characteristic crystals for the purpose. These were either allowed to crystallize out on a coverslip placed in the crystallizing dish, or were scraped up and placed upon it with a clean scalpel.

Enzyme block. Loss of pyruvic acid might be expected, principally from the activity of carboxylase [James & Norval, 1938]. Barley saps as used in the present experiments released CO_2 from added pyruvic acid. The release was slow and was shown, in another series of experiments, to be much less than when solid leaf material was present.

Low concentrations of 1-naphthol-2-sulphonic acid were used to restrict this carboxylase activity. Complete inhibition was not attempted, since the necessary concentration of the naphtholsulphonic acid caused heavy precipitations in the sap. High percentage yields of pyruvic acid could not, therefore, be expected, and were not obtained. Losses due to dephosphorylation of substrates by phosphatases are also known to occur [James & Heard, 1940].

Experiments

Sap only. (*a*) *Exp. 48.* A sample sap prepared as above was precipitated with *N*/5 barium acetate (1 part acetate to 20 parts sap). The precipitate was centrifuged off and the excess barium removed with a few drops of saturated ammonium sulphate. 5 ml. clear filtrate were added to 1 ml. 0·2 % 1-naphthol-2-sulphonic acid, and made up to 10 ml. with glass-distilled water. Crushed thymol (3 parts per 1000) was shaken with the digest which was put in a conical flask plugged with cotton-wool and incubated at 30° for 48 hr. No alkali-soluble 2:4-dinitrophenylhydrazone could be isolated.

(*b*) *Exp. 70A.* A sample of sap from young and vigorously growing seedlings was prepared in the usual way and was used immediately, without either barium precipitation or standing at low temperature for 24 hr. 40 ml. were mixed with 5 ml. 1 % sulphonic acid and 5 ml. glass-distilled water. Crushed thymol (3 per 1000) was added and incubation carried out as before. On extraction by the usual method, a small quantity of a 2:4-dinitrophenylhydrazone, soluble in alkali, was obtained. It was red in colour and crystallized poorly, but a narrow zone of spindle-shaped crystals was found which gave a rather indefinite M.P. 205–208°. These results suggest that small amounts of hydrazone-forming substances had been isolated, probably including traces of pyruvic acid. Since it was already known that under suitable conditions barley could be induced to accumulate pyruvic acid, no attempt was made to obtain definite yields in these digests. The absence of pyruvic acid from freshly extracted saps has already been shown [James & Norval, 1938; James & James, 1940].

Sucrose. Exp. 50. Incubation under the usual conditions was carried out with 24 ml. sap, 3 ml. 20 % sucrose (B.D.H. Analar) and 0·3 ml. 0·2 % sulphonic acid. Crushed thymol was added. The sap used in this experiment was precipitated with barium, as described under (*a*) above. This treatment might be expected to reduce the concentration of phosphate, free and combined, in the sap. No trace of alkali-soluble hydrazones could be isolated.

Glucose and fructose. Special experiments with additions of glucose and fructose were not carried out, since these sugars were known to be present in fresh sap in fair quantity. Their presence did not, in itself, lead to the formation of appreciable quantities of pyruvic acid.

Adenylic acid. Exp. 68B. This experiment was carried out with sap from young green seedlings. The sap was filtered and allowed to stand at 2° for 3 days before use. Its sugar content might be expected to be low. 40 ml. sap were incubated for 48 hr. at 30° with 5 mg. adenylic acid and 0·36 ml. 3 % 1-naphthol-2-sulphonic acid, in the presence of thymol (3 per 1000). It was cleared and distilled and the 2:4-dinitrophenylhydrazones formed in the usual way. The yield was small, with a narrow zone of canary-yellow crystals, probably the pyruvic acid derivative, round the edge. The bulk of the product was orange-coloured and oily. The result was markedly similar to that obtained with fresh sap only (see (*b*) p. 589).

Sucrose + adenylic acid. Exp. 68A. This experiment was carried out simultaneously with the adenylic acid experiment, with an equal aliquot of the same sap. All conditions were identical, except for the addition of 1 g. sucrose to this second digest. A much increased yield of alkali-soluble hydrazone was obtained which crystallized out wholly, though the last crystals to form were somewhat orange in colour. There were numerous bright yellow crystals of the spindle to diamond shape characteristic of the pyruvic acid hydrazone. They gave a deep red colour with alcoholic KOH, which was not discharged on adding water. M.P. 218°, corrected.

Glucose + adenylic acid. Exp. 69. A further supply of sap was prepared exactly as before and used after 24 hr. standing at low temperature. It was possible to use a rather larger digest: 70 ml. sap + 5 mg. adenylic acid + 1·75 g. glucose (B.D.H. analar) + 0·63 ml. 3 % 1-naphthol-2-sulphonic acid + thymol (3 per 1000). The result was similar to that of the previous experiment. A good yield was obtained of characteristic canary-yellow crystals, slightly contaminated with orange, which gave M.P. 216·5°, corrected, and the usual colour reaction with KOH.

Hexosediphosphate. A commercial sample of barium hexosediphosphate containing inorganic phosphate as impurity was used. The ratio of unhydrolysable P to P hydrolysed in 3 hr. was 0·64 as compared with 0·66 calculated from Lohmann's analyses of purified hexosediphosphate. We are indebted to Mr C. R. C. Heard for these figures. Immediately before use, aliquots of the barium salt were dissolved in dilute HCl and the barium centrifuged off after precipitation by a little saturated ammonium sulphate. The solution was neutralized (purple to litmus) with a few drops of strong KOH and made up to volume.

Exp. 47. A preliminary experiment was carried out using 20 ml. of clarified sap (as in (*a*) p. 589) + 4 ml. of a hexosediphosphate solution equivalent to 0·4 g. barium salt + 4 ml. 0·2 % 1-naphthol-2-sulphonic acid + crushed thymol (3 per 1000). The digest was made up to 40 ml. with glass-distilled water. Incubation was carried out under the usual conditions and hydrazones were prepared. There was a small yield of bright yellow crystals, some developing typical diamond forms. They gave a permanent red colour with Lu's [1939] reagent (6 vol. 10 % Na_2CO_3 + 4 vol. *N* NaOH). There was not sufficient for the determination of a melting-point by the usual laboratory method, and the hot-box for micro-determinations had not been prepared when this experiment was carried out. A melting-point was, therefore, not obtained; but there seems little doubt that a small quantity of pyruvic acid had been isolated. Since the clarified sap used in this experiment gave an entire blank when incubated alone (p. 589), it is reasonable to assume that the pyruvic acid originated from the added hexosediphosphate. Confirmation was afforded by an experiment on a rather larger scale.

Exp. 54. 40 ml. sap + 10 ml. hexosediphosphate solution (≡0·1 g. Ba hexosediphosphate) + 6 ml. 0·2 1-naphthol-2-sulphonic acid + thymol were incubated for 48 hr. at 30°. Isolation of alkali-soluble hydrazones was carried out as usual, yielding a considerable deposit of crystals, mostly canary-yellow, though a little reddish in places. Alcoholic KOH gave a permanent deep red colour which was not discharged on the addition of water. The abundant deposit was largely 'run together' but good diamond-shaped platelets were formed in the sparser zones. These gave M.P. 216°, corrected.

Phosphoglycerate. A pure preparation of the crystalline acid barium salt was given us by Prof. R. A. Peters, to whom we wish to express our gratitude. It was brought into solution in the same way as the hexosediphosphate, immediately before use.

Exp. 67. This experiment was carried out using an aliquot of the same lot of sap as was used for the adenylic acid and adenylic acid + sucrose experiments. 4 ml. phosphoglycerate solution (≡30 mg. BaH_2 phosphoglycerate) and 0·72 ml. 3 % 1-naphthol-2-sulphonic acid were made up to 1000 ml. with sap. After the usual incubation, the preparation and isolation of alkali-soluble hydrazones were carried out, yielding a deposit of yellow crystals. There were crystal clusters and single crystals of typical pyruvate form, giving the usual permanent red colour with KOH; M.P. 216°, corrected.

Lactic acid. Lactic acid is not oxidized by barley saps alone, but on addition of ascorbic acid rapid oxidation takes place [James & Cragg, 1940]. The product of this oxidation was identified as pyruvic acid by means of the above technique, slightly modified.

Exp. 38. 200 ml. sap, clarified as in (*a*), were incubated with 40 ml. *N*/5 lactic acid + 40 ml. *N*/10 ascorbic acid (B.D.H.) + 4 ml. sulphonic acid + 116 ml. phosphate buffer, *p*H 7·0 (=400 ml. in all). Incubation lasted 17 hr. at 30°. The digest was then killed with 5 % trichloroacetic acid. After it had stood overnight in a refrigerator, 25 % trichloroacetic acid was added to a final concentration of 5 %. After further standing, the digest was brought to the boil and distilled. A fraction coming over at 50–55° and 20 mm. was taken and treated with an amount of 2:4-dinitrophenylhydrazine, which proved to be inadequate. When the hydrazone was finally separated it was oily, and there was a strong smell of free pyruvic acid. Further hydrazine was added and a plentiful precipitate of yellow crystals was finally obtained. They had typical pyruvate forms, gave deep red coloration with alcoholic potash and had M.P. 216°, corrected.

Inhibition of pyruvate formation by sodium fluoride

Exps. 47 and 54 above show that barley saps convert hexosediphosphate into pyruvic acid. Phosphoglycerate and lactate are also converted into pyruvate and either might, in theory, be an intermediate in the conversion of hexosediphosphate into pyruvate. The reaction phosphoglycerate → pyruvate, or, more exactly, its partial reaction 2-phosphoglycerate ⇆ phosphopyruvate is markedly sensitive to poisoning by sodium fluoride. Inhibition of the hexosediphosphate → pyruvate reaction by sodium fluoride would therefore suggest conversion via phosphoglycerate, whereas tolerance of this poison would rule it out.

Exp. 70. A fresh preparation of sap was divided into 3 aliquots of 40 ml. each and the usual amounts of sulphonic acid and thymol were added.

A was made up to 50 ml. with glass-distilled water;

B with a solution of hexosediphosphate (≡40 mg. Ba hexosediphosphate);

C with equivalent hexosediphosphate and NaF to a final concentration of $M/40$.

The three digests were incubated side by side for 48 hr. at 30° and alkali-soluble hydrazones isolated in the usual way. The results may be summarized as follows:

Table 1

	Digest	Distillation	Products
A	Sap + water	Complete below 50°	Partly crystalline, reddish. Narrow zone spindle-shaped crystals
B	Sap + hexosediphosphate	Temp. rose above 50°	Much larger and yellower deposit than A
C	Sap + hexosediphosphate + $M/40$ NaF	Complete below 50°	Quantity and colour very similar to A

The experiment suggested a marked inhibition due to sodium fluoride, but was necessarily inconclusive because the amount of product could only be determined by inspection, and because it was found that amounts of pyruvic acid added to sap could not be quantitatively recovered by the distillation. We therefore sought for means of estimating pyruvic acid formation more exactly. Lu's [1939] method, utilizing the colour of the hydrazone in strongly alkaline solution, was found to be useless because of the presence of varying amounts of other substances in the sap which gave intense colours and which could not be separated without serious loss of pyruvate. Straub's [1936] *o*-hydroxybenzylidene-pyruvate method suffered from the same disadvantage when applied to barley sap, but occasionally a sample of sap was obtained in which the interfering substances were present in relatively small amounts. Graded additions of pyruvic acid could then be added and a calibration graph constructed for that particular sap. This proved extremely laborious, since many samples of sap had to be examined before a suitable one was found. The first two suitable saps obtained gave results which confirmed the suggestion of the qualitative experiment above.

Exps. 56 and 63. The sap was prepared in the usual way, and the digests were set up as described below (see Table 2): Digest A was fixed and estimated immediately. Digests B and C were incubated for 46 hr. in the presence of thymol; they were then cleared with barium and trichloroacetic acid by the same method as was used in the isolation experiments, and each digest was made up to 10 ml. Aliquots of 4 ml. were taken from these and 4 ml. of salicylic aldehyde in alcohol and 2 ml. of 166 % KOH were added. The red colour of *o*-hydroxy-benzylidene pyruvate was then developed by warming at 37° for 10 min., cooling and centrifuging exactly as described by Straub. The colorimetry was carried out with a Spekker absorptiometer, using a No. 6 (blue) screen. The results are given as arbitrary readings from the instrument, since the colour of the controls is not due to pyruvic acid. The assumption that the colour produced during incubation is in the main due to pyruvic acid is based on the results of the isolation experiments. The instrument numbers rise with increasing concentration of the coloured compound, though not necessarily in strict proportion.

Table 2

	Sap ml.	Hexosedi-phosphate ml.	1-Naphthol-2-sulphonic acid ml.	NaF ml.	Water ml.	Readings Exp. 56	Readings Exp. 63
A	2	1		—	1·0	322	298
			0·5	—	0·5	—	—
B	2	1	0·5	—	0·5	444	429
C	2	1	0·5	0·5	—	297	399

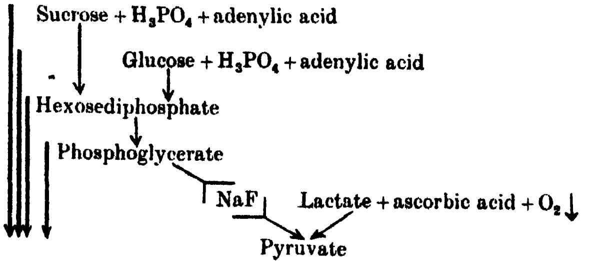
Final concentrations: NaF $= M/40$, 1-naphthol-2-sulphonic acid $= 0{\cdot}1$ %. In Exp. 56 the hexosediphosphate added was equivalent to 3 mg. pyruvic acid and, making the necessary assumptions, the pyruvic acid estimated after incubation of digest B was about 1·2 mg.

In both the experiments the presence of NaF (digest C) has led to a smaller production of 'pyruvic acid' than occurred in its absence (digest B). In Exp. 63, inhibition was only partial at the concentration used, but in Exp. 56, at the same concentration, it was complete.

DISCUSSION

It is a commonly held belief that the mechanism of glycolysis in the higher plants is similar to that of alcoholic fermentation in yeast. The experiments described above show that barley saps are able to promote many of the reactions now believed to occur in fermentation. They do not, strictly speaking, show that these reactions occur normally in the living barley plant, but the corresponding evidence is rather scanty, even for yeast. We have, however, shown by other methods (papers quoted in the introduction) that some at least of these reactions are likely to be taking place in living barley tissues. Our experiments were all carried out aerobically and a final formation of alcohol was, therefore, not to be expected. The exact effect of oxygen is reserved for future consideration, but it is a matter of interest that these glycolytic reactions would take place in its presence.

The reactions we have actually shown to be catalysed by barley sap are indicated by the heavy side arrows in the following diagram. The formation of pyruvate from lactate differs from the other reactions in consuming oxygen [James & Cragg, 1940], whereas they only tolerate it.


Sucrose + H_3PO_4 + adenylic acid
Glucose + H_3PO_4 + adenylic acid
Hexosediphosphate
Phosphoglycerate
NaF
Lactate + ascorbic acid + O_2
Pyruvate

The following remarks apply to the glycolytic reactions in barley systems.

Sucrose → pyruvate. In saps with low phosphate concentrations, this reaction fails to occur [Exp. 50], but it may be brought about in saps with a normal content of inorganic phosphate by the addition of adenylic acid [Exp. 68A]. Adenylic acid has not yet been isolated from barley, but the presence of a labile phosphate carrier is suggested by the fact that the organic esters contain a small readily hydrolysable fraction [Arney, 1939]. This small amount might be rendered inoperative in the extracted saps by dilution.

Glucose → pyruvate. This reaction presents the same general characteristics as the sucrose → pyruvate reaction, i.e. it will only occur if means are provided for phosphorylation [Exp. 69]. It is therefore a reasonable assumption that both sugars are converted into phosphate esters before their actual splitting. Barley saps always contain a relatively large amount of free phosphate unless special steps are taken for its removal. We do not at present know whether this is continuously drawn upon for phosphorylation, or whether a closed cycle, excluding free phosphate, is established.

Hexosediphosphate → pyruvate. It is probable that a plexus of sugar-phosphate esters occurs in barley as in other plants and organisms. Hexosediphosphate is likely to be one of the esters and probably the most abundant. This is indicated by analysis of the phosphate fractions by the usual methods [James & Heard, 1940]. Added hexosediphosphate is decomposed [James & Heard, 1940] with formation of pyruvate by barley saps (Exps. 54, 56, 63, 70) without addition of adenylic acid or any other agent, even when free inorganic phosphate has been precipitated beforehand (Exp. 47). Inhibition of this reaction by $M/40$ NaF (Exps. 56, 63, 70) suggests that its course lies by way of phosphoglycerate and phosphopyruvate. Another probable stage, that involving triosephosphate, is not considered here, since the evidence collected depends on other methods.

Phosphoglycerate → pyruvate. Phosphoglycerate has not yet been isolated from barley. Saps breaking down hexosediphosphate in the presence of NaF accumulate a phosphate ester highly resistant to acid hydrolysis which is likely to be phosphoglycerate [James & Heard, 1940]. Added phosphoglycerate in the absence of NaF gave pyruvate (Exp. 67).

SUMMARY

1. Cell-free barley saps were prepared by a simple method. On incubation for 48 hr. at 30° in the presence of thymol and 1-naphthol-2-sulphonic acid, they gave rise to little or no pyruvic acid.

2. Addition of sucrose did not cause pyruvic acid formation. Glucose and fructose would be present in the sap itself, and therefore were also incapable of giving pyruvic acid.

3. Addition of adenylic acid to starved sap (i.e. sap low in sugar-content) gave traces of what was probably pyruvic acid.

4. Addition of sucrose + adenylic acid, or glucose + adenylic acid, led to the formation of readily identifiable quantities of pyruvic acid.

5. Hexosediphosphate and phosphoglycerate were also converted into pyruvic acid. Pyruvate formation from hexosediphosphate was markedly inhibited by $M/40$ NaF.

6. Lactic acid was oxidized to pyruvic acid in the presence of ascorbic acid and atmospheric oxygen.

These results, 1–5, indicate a conversion of sugars into pyruvic acid through a phosphorylating cycle similar, at least in outline, to the reactions of yeast.

We wish to acknowledge the assistance of Dr Margaret Cattle in the latter part of the work.

REFERENCES

Arney (1939). *Biochem J.* **33**, 1078.
James & Arney (1939). *New Phytol.* **38**, 340.
—— & Cragg (1940). *New Phytol.* **39**, 334.
—— & Heard (1940). *New Phytol.* **39**, 334.
—— & James (1940). *New Phytol.* **39**, 266.
—— & Norval (1938). *New Phytol.* **37**, 455.
Lu (1939). *Biochem. J.* **33**, 249.
Peters & Thompson (1934). *Biochem. J.* **28**, 916.
Straub (1936). *Hoppe-Seyl. Z.* **244**, 117.

ERRATUM

Vol. 35, Nos. 1 & 2, p. 46

for $CH_2OH.CH_2NH_2 + IO_4^- \rightarrow IO_3^- + HCHO + H_2O + NH_3$
read $CH_2OH.CH_2NH_2 + IO_4 \rightarrow IO_3^- + 2HCHO + NH_3$

62. THE ENZYMIC DECARBOXYLATION OF OXALOACETATE

By LESTER ORVILLE KRAMPITZ and
CHESTER HAMLIN WERKMAN

From the Bacteriology Section, Industrial Science Research Institute, Iowa State College, Ames, Iowa

(*Received 21 April 1941*)

Although oxaloacetate has an important role in cellular metabolism, no enzyme has been described which brings about a rapid decarboxylation of the acid. Breusch [1939] investigated a heat-stable component of mammalian tissue responsible for this decarboxylation but could not identify it with any known physiological agent. The rate of this decarboxylation is comparatively slow. In investigations on the utilization of CO_2 by bacteria, Wood & Werkman [1938; 1940] postulated the reverse reaction, i.e. the carboxylation of pyruvate to form oxaloacetate. This reaction was represented as follows:

$$CO_2 + CH_3.CO.COOH \rightarrow HOOC.CH_2.CO.COOH.$$

Evans & Slotin [1940] have demonstrated the utilization of CO_2 with pigeon liver by the use of radioactive CO_2. The mechanism suggested by them is essentially the Wood & Werkman reaction. Krebs & Eggleston [1940], also working with pigeon liver, suggested that the reaction occurs in the dissimilation of pyruvate. They postulate that aneurin is required in the fixation. Smyth [1940] came to the same conclusion using vitamin-deficient staphylococci.

It is the purpose of the present communication to report the occurrence of a thermolabile enzyme which brings about the decarboxylation of oxaloacetate to pyruvate and CO_2. Experimental evidence is presented to show that (1) the magnesium ion and not cocarboxylase is a coenzyme for the enzyme; (2) the products of decarboxylation are pyruvate and CO_2; (3) the enzyme is heat-labile and (4) permeability of the cell wall is an important factor to be considered in studies on the enzyme. Furthermore, it is believed that under proper experimental conditions the enzyme described is capable of bringing about the carboxylation involved in CO_2 utilization.

METHODS

Micrococcus lysodeikticus was employed in the investigation. The organisms were grown on a medium containing 1 % glucose, 0·5 % peptone, 0·3 % yeast extract, 10 % tap water and 2 % agar. Inoculations were made on agar slopes in large Roux bottles. After 3 days' incubation at 30°, the cells were washed from the agar surface with distilled water, filtered through glass wool and washed twice in a centrifuge.

Acetone-treated cells. 10 g. of cell paste were mixed with 70 ml. ice-cold acetone and shaken thoroughly for 5 min. The cells were filtered off, suspended in 140 ml. ice-cold acetone and shaken vigorously for 10 min. This process was repeated. The preparation was then placed *in vacuo* to remove traces of acetone. These cells are permeable to oxaloacetate, whereas untreated cells are not.

Lysed cell preparation. To a 10 % cell suspension (wet weight basis), one-tenth volume of saliva was added, and the mixture incubated 1 hr. at 36°, whereupon lysis occurred. For control, a 10 % cell suspension without saliva was incubated in like manner.

Cocarboxylase- and Mg^{++}-deficient acetone-treated cells. The usual alkaline phosphate washing was employed. 1 g. of acetone-treated cells was washed twice for 10 min. on the shaking machine with 50 ml. phosphate buffer, *p*H 8·0, and once with 50 ml. distilled water. The cells maintain their deficiency if dried on a porous plate. Cocarboxylase and Mg^{++} cannot be removed by phosphate washing unless the cells are first treated with acetone.

Cocarboxylase- and Mg^{++}-deficient preparation of lysed cells. To 4 ml. of a 20 % solution of lysed cells, 6 ml. of saturated $(NH_4)_2SO_4$ were added. The mixture was vigorously shaken for 10 min. and centrifuged. The precipitated particles were made up to a volume of 4 ml. with distilled water and the $(NH_4)_2SO_4$ washing was repeated twice. Finally, the precipitate was washed with distilled water and made up to the original volume of 4 ml.

Preparation of oxaloacetic acid. To the Na salt of diethyloxaloacetate (100 g. in 250 ml. water) conc. H_2SO_4, equivalent to the Na, was added. The free ester was removed as an oily layer by means of a separating funnel and dissolved in 80 ml. ether. The ether solution was washed twice with 50 ml. distilled water, and the ether evaporated off. The ester was hydrolysed by treatment with 4 vol. conc. HCl at room temperature and vigorously shaking the acid solution mechanically for at least an hour. The solution was then placed at −26° for 24–48 hr. The precipitated acid was removed by filtration, dissolved in the requisite amount of warm acetone and recrystallized by adding chloroform. The purity of such a sample as determined by the aniline citrate method [Ostern, 1933] is greater than 98 %.

Pyruvic acid was determined by the carboxylase method [Krebs *et al.* 1940] and also by the salicylaldehyde method of Straub [1936]. CO_2 was determined by adding 0·3 ml. of 1 : 1 H_2SO_4 to the reactants in the manometer vessel.

The Barcroft-Warburg respirometer was used in this study. The total volume of reactants in each cup was 2 ml. Experiments conducted with untreated cells showed an endogenous activity which was deducted from values obtained with the substrates present. Endogenous activity of acetone-treated cells was negligible.

EXPERIMENTAL

Permeability. The untreated cells of *M. lysodeikticus* aerobically oxidized pyruvate, oxaloacetate, malate, fumarate and succinate with almost equal rapidity (Table 1). Anaerobically there was no evolution of CO_2 from any of these salts. The acetone-treated cells behaved similarly to the untreated cells excepting that they oxidized succinate very slowly and decarboxylated oxaloacetate very rapidly anaerobically. Since there was no increase in CO_2 evolution in the presence of the untreated cells and oxaloacetate, it may be assumed that the oxaloacetate did not reach the active enzyme centres. Apparently, the acetone extraction of the cells makes the cell permeable to oxaloacetate. Objections to this explanation may be made, inasmuch as the untreated cells showed an O_2-uptake with oxaloacetate. It is probable, however, that the oxaloacetate did not penetrate the untreated cell even in the aerobic experiments, and that the O_2-uptake was due to the oxidation of pyruvate formed by the spontaneous decarboxylation of oxaloacetate. This explanation is supported by the fact that anaerobically with the acetone-treated cells, the quantity of

Table 1. *Anaerobic and aerobic behaviours of* Micrococcus lysodeikticus *on the C_4 dicarboxylic acids and pyruvate*

	Untreated cells		Acetone-treated cells		
Substrate	Aerobic μl. O_2 uptake	Anaerobic μl. CO_2 evolved	Aerobic μl. O_2 uptake	Aerobic μl. CO_2 evolved	Anaerobic μl. CO_2 evolved
Pyruvate	248	6	262	320	12
*Oxaloacetate	225	4	255	1220	915
Malate	250	2	298	—	3
Fumarate	228	3	314	—	2
Succinate	240	1	12	—	0

Total volume of reactants = 2 ml. Conc. of substrates 0·025 *M*, 20 mg. cells per cup (dry weight), 0·125 *M* PO_4 buffer *p*H 6·8; aerobic exp. contained air in gas space, anaerobic contained N_2. Temp. 30°. Time 1 hr.

* All values corrected for spontaneous decarboxylation of oxaloacetate. Endogenous values deducted in experiments containing substrates.

CO_2 from oxaloacetate (915 μl.) is substantially equivalent to the CO_2 from the corresponding aerobic experiment (1220 μl.) minus the CO_2 from the aerobic pyruvate experiment (320 μl.). Apparently the excess CO_2 in the aerobic experiment is the result of pyruvate dissimilation. Pyruvate is not dissimilated anaerobically; consequently the CO_2 evolved from oxaloacetate under these conditions must have arisen by its decarboxylation, shown by the 915 μl. of CO_2 obtained anaerobically from 928 μl. of oxaloacetate.

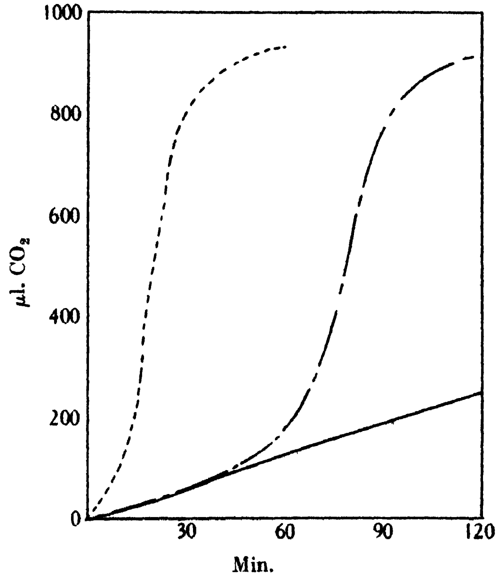


Fig. 1. Effect of lysis on the decarboxylation of oxaloacetate. Total volume of reactants 2·0 ml. Anaerobic; 0·125 *M* PO_4 buffer *p*H 6·8, temp. 30°. —— 2·5 % untreated cells + 920 μl. oxaloacetate. ---- 2·5 % lysed cells + 920 μl. oxaloacetate. ---- 2·5 % untreated cells + 0·1 volume saliva + 920 μl. oxaloacetate.

Objections may also be raised that if the other C_4 acids reach their enzyme centres, oxaloacetic acid should likewise do so. Apparently other compounds having the grouping —CO.CH_2.CO— do not penetrate the cell wall, e.g. malonic acid, generally recognized as an inhibitor of succinate oxidation, shows no inhibition with *M. lysodeikticus*, even after long incubation before the succinate addition. Apparently, the malonate does not penetrate the cell to the enzyme centre. This hypothesis cannot be tested with our acetone preparation, inasmuch as it does not oxidize succinate (Table 1).

Fig. 1 further substantiates the cell permeability hypothesis. *M. lysodeikticus*, as shown by Fleming [1924], is lysed by lysozyme, an enzyme present in both egg albumin and saliva. In the figure the decarboxylations of oxaloacetate by untreated cells, by previously lysed cells and by cells that were lysed during the course of the experiment are compared. If cell permeability is limiting the decarboxylation of oxaloacetate, it is to be expected that as lysis proceeds the enzyme will become liberated and be available for decarboxylation. Decarboxylation by the untreated cell was identical with the spontaneous decarboxylation; therefore the two curves are superimposable. The lysed preparation showed a remarkable ability to decarboxylate oxaloacetate, and the experiment in which lysis proceeded in the presence of oxaloacetate showed an increasing rate of decarboxylation. Saliva alone does not increase the spontaneous decarboxylation. The results of these experiments clearly establish the importance of cell permeability.

Products of the decarboxylation of oxaloacetate. Pyruvic acid and CO_2 are formed by the anaerobic breakdown of oxaloacetate (Table 2). For each oxaloacetic acid molecule decarboxylated, one molecule of pyruvate and one of CO_2 were found. The pyruvate determinations by both the carboxylase and the salicylaldehyde methods agreed, and there was an equivalent recovery of CO_2 on acidification. The oxaloacetate was determined by the aniline citrate method.

Table 2. *Products of anaerobic decarboxylation of oxaloacetate by an acetone preparation*

Material determined	Method of determination	Quantity obtained (μl.)	Recovery %
Oxaloacetate dissimilated	Aniline citrate	998	—
Pyruvate	Carboxylase	958	97
Pyruvate	Salicylaldehyde	970	98
CO_2	Acidification	947	96

Effects of Mg^{++} and cocarboxylase on the enzyme. The question next arises as to the relationship between oxaloacetate carboxylase and pyruvate carboxylase. Krebs & Eggleston [1940] and Smyth [1940] are of the opinion that the two are either identical or very similar. It is well known that yeast carboxylase not only decarboxylates pyruvate but also oxaloacetate. The products of decarboxylation of the latter are acetaldehyde and two molecules of CO_2. There are two possible mechanisms by which yeast carboxylase may decarboxylate oxaloacetate: initial β-decarboxylation or initial α-decarboxylation.

β-decarboxylation

(1) $HOOC.CH_2.CO.COOH \rightarrow CH_3.CO.COOH + CO_2$
$CH_3.CO.COOH \rightarrow CH_3.CHO + CO_2$

α-decarboxylation

(2) $HOOC.CH_2.CO.COOH \rightarrow HOOC.CH_2.CHO + CO_2$
$HOOC.CH_2.CHO \rightarrow CH_3.CHO + CO_2$ (spontaneous).

To obtain information relative to this question, the Mg^{++} and the cocarboxylase requirements of the two reactions have been determined. It is well known that in both the aerobic and the anaerobic decarboxylations of pyruvic acid, cocarboxylase and Mg^{++} are required. With *M. lysodeikticus* it was found that the organism likewise requires cocarboxylase and Mg^{++} for the α-decarboxylation. On the contrary, for the β-decarboxylation of oxaloacetate only Mg^{++} is required. The difference in the coenzyme requirements indicates that the two enzymes are not alike. Whether decarboxylation of oxaloacetate by yeast involves two enzymes or occurs solely by α-decarboxylation must await further investigation. The present investigation shows that with bacteria the former is the case.

Data in Table 3 show the results with acetone-treated cells made deficient in cocarboxylase and Mg^{++}. Deficiencies were determined by observing the lack of ability of the preparation to oxidize pyruvate and oxaloacetate aerobically.

Table 3. *Pyruvate and oxaloacetate metabolism by Mg^{++} and cocarboxylase deficient and non-deficient acetone-treated cells*

	Deficient cells plus.						
Substrate	No addition	Mg^{++}	Cocarboxylase	Mg^{++} and cocarboxylase	Mn^{++}	Mg^{++} and thiamin	Non-deficient acetone-treated cells
Pyruvate, μl. O_2 uptake	14	16	17	212	18	18	250
*Oxaloacetate, aerobic, μl. O_2 uptake	18	20	19	219	22	22	255
*Oxaloacetate, aerobic μl. CO_2 formed	41	927	38	1206	932	926	1228
*Oxaloacetate, anaerobic, μl. CO_2 formed	32	930	30	930	934	926	928

Total volume of reactants 2 ml. 0·025 *M* oxaloacetate, 0·006 *M* $MgCl_2$, 0·006 *M* $MnSO_4$, 25 μg. cocarboxylase or thiamin, 20 mg. cells per cup (dry weight), 0·125 *M* PO_4 buffer *p*H 6·8; aerobic exp. contained air in gas space, anaerobic contained N_2. Temp. 30°. Time 1 hr.

* Spontaneous decarboxylation values of oxaloacetate deducted from substrate values. With Mg^{++} 178 μl. Without Mg^{++} 94 μl.

Upon addition of Mg^{++} and cocarboxylase to the deficient cells, the oxidation of pyruvate took place. 212 μl. O_2 were taken up with cocarboxylase and Mg^{++} addition, and only 14 μl. with no addition. The non-deficient acetone-treated cells took up 250 μl. O_2. When either Mg^{++} or cocarboxylase alone was added, no oxidation occurred. Therefore, it is apparent that both Mg^{++} and cocarboxylase are essential for pyruvic acid oxidation. The addition of aneurin and Mg^{++} had no effect, indicating that the mechanism for phosphorylating the vitamin had been destroyed by the acetone treatment.

The difference between pyruvate and oxaloacetate decarboxylations is apparent if the effects of Mg^{++} and cocarboxylase on oxaloacetate dissimilation are considered. Considering O_2 uptake alone, the difference is not evident, since both Mg^{++} and cocarboxylase are required for O_2 utilization. This oxidation does not involve oxaloacetate but pyruvate arising from the decarboxylation of the oxaloacetate. The picture, however, is different with respect to CO_2 evolution. The deficient cells were unable to decarboxylate oxaloacetate, e.g. only 41 μl. CO_2 were liberated. The addition of Mg^{++} restored both the anaerobic decarboxylation (930 μl.) and the aerobic decarboxylation (927 μl.) to the original values, which were comparable with those found when both Mg^{++} and cocarboxylase were added (930 μl. anaerobically and 1206 μl. anaerobically). Obviously cocarboxylase is not required for the decarboxylation of oxaloacetate. Mg^{++} increased the spontaneous decarboxylation of oxaloacetate and proper corrections were made.

In Table 4 data are given which were obtained with a lysed deficient preparation. With this preparation it should be stated that the dehydrogenase activity had been almost destroyed by lysing; consequently there was little O_2 uptake with pyruvate. This is in accordance with the findings of Penrose & Quastel [1930]. Values representing this O_2 uptake, therefore, have been omitted from the Table. The oxaloacetate decarboxylation values, however, show substantially the same results as those obtained with the acetone-treated preparation.

Table 4. *Metabolism of deficient and non-deficient lysed cells with oxaloacetate*

	Deficient lysed cells plus						
Substrate	No addition	Mg^{++}	Cocarboxylase	Mg^{++} and cocarboxylase	Mn^{++}	Mg^{++} and aneurin	Non-deficient lysed cells
*Oxaloacetate, anaerobic, µl. CO_2	52	928	48	934	927	922	917
*Oxaloacetate, aerobic, µl. CO_2	54	925	49	916	922	918	920

Total volume of reactants 2 ml. 0·025*M* oxaloacetate, 0·006*M* $MgCl_2$, 0·006*M* $MnSO_4$, 25 µg. cocarboxylase or aneurin, 20 mg. cells per cup (dry weight), 0·125*M* PO_4 buffer *p*H 6·8; aerobic exp. contained air in gas space, anaerobic contained N_2. Temp. 30°. Time 1 hr.

* Spontaneous decarboxylation of oxaloacetate values deducted. With Mg^{++} 178 µl. Without Mg^{++} 94 µl.

Effect of heat on the enzyme. It is well known that there is a heat-stable component present in mammalian tissue that activates the decarboxylation of oxaloacetate [Breusch, 1939]. The heat stability of our enzyme therefore has been studied.

That our preparation is not the heat-stable component is shown by Table 5. These results indicate that the enzyme is heat-labile and is almost completely destroyed at 100° in 5 min. The small value obtained under these conditions may be due to the same component discussed by Breusch.

Table 5. *Effect of heat on enzyme*

	Lysed preparation				Acetone preparation	
Substrate	No heat	60° 10 min.	80° 10 min.	100° 5 min.	No heat	100° 5 min.
*Oxaloacetate, anaerobic, µl. CO_2	944	498	40	15	948	30

0·025*M* oxaloacetate, 0·125*M* PO_4 buffer *p*H 6·8, 2·5% lysed preparation per cup; 20 mg. acetone preparation per cup. Gas space N_2. Total volume of reactants 2·0 ml. Temp. 30°. Time 1 hr.

* Spontaneous decarboxylation values deducted.

DISCUSSION

The results reported here appear to differ from those reported by Smyth [1940] and Krebs & Eggleston [1940]. These investigators believe that aneurin is necessary for the synthesis of oxaloacetate from pyruvate and CO_2. Smyth, working with staphylococci, showed that oxaloacetate could replace aneurin in the dismutation of pyruvate. He concluded that aneurin is required for the synthesis of oxaloacetate, which acts as a hydrogen acceptor in the dismutation of pyruvate to lactate, acetate and CO_2. Therefore in the presence of oxaloacetate the vitamin is not necessary for the dismutation. If this were the case, our vitamin-deficient acetone preparation should oxidize pyruvate in the presence of oxaloacetate. This was not true in our experiments, since O_2-uptake and decarboxylation of pyruvate are obtained only on addition of Mg^{++} and cocarboxylase to the oxaloacetate experiments. It must be borne in mind that with oxaloacetate as the substrate, there is a sufficient supply of pyruvate at all times, owing to spontaneous decarboxylation of oxaloacetate. It should be pointed out that the results of Smyth are by no means conclusive proof of the claimed function of the vitamin. Although the anaerobic dissimilation of pyruvate has not been studied as thoroughly as the aerobic oxidation, it is probable that the component parts of the system are very similar. In the aerobic dissimilation of pyruvate [Lipmann, 1939; Banga *et al.* 1939], cocarboxylase, a C_4 dicarboxylic acid, inorganic phosphate, adenine nucleotide and Mg^{++} are components of the system. The oxidation thus requires cocarboxylase even in the presence of C_4 acids. Certainly cocarboxylase, in this case, has a function other than in the synthesis of C_4 acids. The system investigated by Smyth is very complex and there is a probability of a multiple deficiency. The removal of any one of these deficiencies might stimulate the reaction. The speed of the reaction may be limited by an additional factor when either aneurin or C_4 acids or both are added. Particularly in view of our findings, it seems that there may be another explanation of Smyth's results.

Krebs & Eggleston [1940] investigated the dissimilation of pyruvate by pigeon liver and found an increased pyruvate disappearance in the presence of $NaHCO_3$ and CO_2. They concluded that the pyruvate was used in CO_2 fixation, forming oxaloacetate which then undergoes reactions of the citric acid cycle. Aneurin was concluded to be concerned with the fixation reaction because the vitamin stimulated pyruvate dissimilation by liver from an avitaminous pigeon. This stimulation was not obtained by employing breast muscle tissue from the same bird. They concluded that the vitamin acts in a reaction occurring in the dissimilation by liver but not in that by breast muscle. Furthermore, since oxaloacetate synthesis was considered to occur in liver but not in breast muscle, the vitamin was thought to act in this synthesis. The suggested function of the CO_2 is in accord with the observations made with bacteria and is attractive particularly in view of the results of Evans & Slotin [1940]. The evidence for the function of the vitamin in this reaction is, however, not conclusive. Krebs & Eggleston presented no proof that the breast muscle was deficient in aneurin. It cannot be assumed that in an animal suffering from vitamin deficiency all of the organs are equally deficient [cf. Ochoa & Peters, 1938]. Thus the liver of an avitaminous pigeon may be limited in its metabolism of pyruvate, whereas the breast muscle may still contain sufficient cocarboxylase for an active reaction. Therefore, the evidence offered by Krebs & Eggleston that aneurin does not function in pyruvate metabolism of breast muscle needs confirmation, since the vitamin activity will not be apparent if the muscle is not deficient, and therefore their conclusion concerning the function of the vitamin in oxaloacetate synthesis may have another explanation.

We have been unable by use of the enzyme from *M. lysodeikticus* to obtain carboxylation of pyruvate. Inasmuch as the ΔF of the fixation reaction is positive, and therefore requires energy from a coupled reaction, the vitamin may be required in this second reaction. The possibility exists that in our preparation conditions were such that the enzyme catalysing the energy-yielding reaction has been destroyed, and consequently we obtain only the decarboxylation reaction. We are at present attempting to reverse the reaction to obtain carboxylation. It is to be remembered that our preparation was inactive with aneurin but active with the phosphorylated vitamin, indicating that the phosphorylating mechanism has been destroyed. It is probable that the energy-yielding reaction necessary for carboxylation may be coupled with a phosphate exchange which did not occur in our preparation.

SUMMARY AND CONCLUSIONS

An enzyme preparation obtained from *Micrococcus lysodeikticus* is described which catalyses the reaction: $HOOC.CH_2.CO.COOH \rightarrow CH_3.CO.COOH + CO_2$. Magnesium ions are apparently required for the reaction, whereas cocarboxylase and aneurin have no effect. The enzyme is heat-labile, and thus is not the heat-stable compound occurring in mammalian tissue which stimulates the decarboxylation of oxaloacetate. Indications are that the enzyme is required in the carboxylation reaction involved in the CO_2 utilization of Wood & Werkman.

Permeability relationships must be considered in working with oxaloacetate and whole cells of *Micrococcus lysodeikticus*.

REFERENCES

Banga, Ochoa & Peters (1939). *Biochem. J.* **33**, 1980.
Breusch (1939). *Biochem. J.* **33**, 1757.
Evans & Slotin (1940). *J. biol. Chem.* **136**, 301.
Fleming (1924). *Lancet*, **206**, 1303.
Krebs & Eggleston (1940). *Biochem. J.* **34**, 1383.
—— Eggleston, Kleinzeller & Smyth (1940). *Biochem. J.* **34**, 1234.
Lipmann (1939). *Symposia on Quantitative Biology*, **7**, 248.
Ochoa & Peters (1938). *Biochem. J.* **32**, 1501.
Ostern (1933). *Hoppe-Seyl. Z.* **218**, 160.
Penrose & Quastel (1930). *Proc. roy. Soc.* B, **107**, 168.
Smyth (1940). *Biochem. J.* **34**, 1598.
Straub (1936). *Hoppe-Seyl. Z.* **244**, 117.
Wood & Werkman (1938). *Biochem. J.* **32**, 1262.
—— —— (1940). *Biochem. J.* **34**, 7.

63. PROLIFERATION-PROMOTING INTERCELLULAR HORMONES

2. EVIDENCE FOR THEIR PRODUCTION BY LIVING CELLS AS A RESPONSE TO INJURY

By JOHN R. LOOFBOUROW,[1] SISTER CECELIA MARIE DWYER, S.C., AND SISTER ANN GERTRUDE CRONIN, S.C.

From the Research Laboratories of Institutum Divi Thomae, Cincinnati, Ohio, and the Research Laboratory of Good Samaritan Hospital, Dayton, Ohio

(*Received 19 May 1941*)

PREVIOUSLY published investigations from these laboratories indicate that when yeast, various other micro-organisms, living tissues of rat, mouse and chicken embryos, and living adult newt tissues are subjected to such varied forms of injury as lethal ultraviolet radiation, X-rays, mechanical injury, chemical irritation and oxygen lack, there appear in the intercellular fluids factors stimulating cellular proliferation [Fardon *et al.* 1937; Norris & Ruddy, 1937; Sperti *et al.* 1937, 1, 2; Loofbourow *et al.* 1938; 1939; 1940]. We have called these factors 'proliferation-promoting intercellular hormones'.[2] Davidson [1940] has recently confirmed our results in the case of proliferation-promoters induced by the irradiation of yeast with lethal ultraviolet.

The possibilities that intercellular hormones from injured cells may act as the initial stimulus in wound healing, and that they may play a part in overgrowth and neoplasia, are obvious reasons for interest in this problem. The conception of a relationship between the products of injured tissues and the repair processes following injury is not new. There is, in fact, an extensive literature regarding this question [for example, see Wiesmer, 1892; Carrel, 1930; Arey, 1936; Menkin, 1940; Loofbourow, 1941]. The techniques employed by our colleagues and ourselves [Sperti *et al.* 1937, 1, 2, 3; Loofbourow *et al.* 1938; 1939; 1940] make possible the controlled comparison of the proliferation-promoting effects of products from injured and uninjured tissues, and hence afford a new quantitative approach to the problem.

In investigating this problem, it seems of importance to distinguish between (*a*) factors released as a result of cell disintegration; (*b*) factors, already in the cell, released as a result of increased membrane permeability; and (*c*) factors synthesized by living, injured cells and released by them into the intercellular fluids. *A priori*, it is conceivable that the proliferation-stimulating activity found in the intercellular fluids of suspensions of injured cells might be attributable entirely to (*a*) or to (*b*) and (*a*). As will appear from the following, however, the experimental evidence seems to indicate that factors of the (*c*) type play a major part in the phenomenon.

---

[1] Present address, Massachusetts Institute of Technology, Cambridge, Mass.

[2] The term 'intercellular hormone' was suggested by Loofbourow & Morgan [1938] for factors from cells which act through the intermediary of the intercellular fluids in affecting the metabolism of the cell community.

EXPERIMENTAL METHODS AND RESULTS

In the following experiments, yeast (*S. cerevisiae*, Fleischmann Bakers' strain) was both the organism injured and the test organism for determining the proliferation-promoting activity of the intercellular fluids. Staining with methylene blue was used to estimate the number of cells killed [Richards, 1932] in the suspensions subjected to injury.

The technique of the growth tests has been described in detail elsewhere [Loofbourow, Dwyer & Morgan, 1938]. The potencies are evaluated in terms of growth units per mg. of dry material or per ml. of fluid, one growth unit being the amount of material (mg. or ml.) which must be added to 1 ml. of culture to cause an increased 24-hr. yeast crop of 1·6 mg./ml. (wet weight) as compared with the controls, under the standard conditions adopted for the growth tests. Since the control crop is normally about 0·16 mg./ml., one growth unit is also (approximately) the quantity of material which must be added to each ml. of culture medium to increase the 24-hr. crop by 1000 %.

1. *Effect of rapid killing compared with that of slow injury.* If proliferation-promoting factors arise in the intercellular fluids primarily as a result of mechanism *a* (disintegration of dead cells), one would expect yields to be nearly the same from the same number of dead cells, whether they were killed rapidly or slowly. If they arise as a result of *b* (increased permeability) or *c* (production of factors by living injured cells), greater yields might be expected from cells killed slowly.

To test this point, yeast was suspended in isotonic salt solution at a concentration of 20 g./l. Aliquots were irradiated with full ultraviolet from a quartz mercury arc for times of 20, 40, 80, 160 and 320 min. The intensities were adjusted (by varying the distances of the samples from the arc) so that approximately 90 % of the cells were dead in each aliquot at the end of its irradiation period. Corresponding control aliquots stood without irradiation for the same lengths of time. Immediately following each irradiation period, the irradiated suspension and the control suspension corresponding to it were filtered through Berkefeld filters, and the cell-free filtrates were taken to dryness. The residues of cells were discarded. For growth tests, the dried products were made up in distilled water to one-fifth their original volume, and assayed by the usual techniques (loc. cit.).

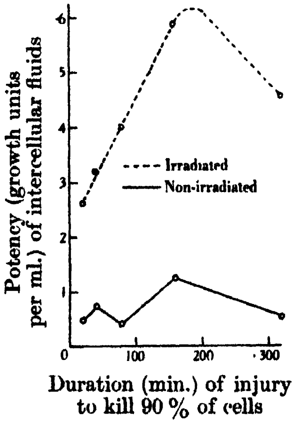


Fig. 1. Effect of decreased rate of killing on increased yield of proliferation-promoting factors.

The results of such an experiment are shown in Fig. 1. In the case of irradiated suspensions, the potency of the extract was approximately a linear function of the duration of injury up to 160 min., after which there was a marked decrease, the cause of which has not been established. Since the same numbers of cells were killed in all instances, these results indicate that the production of active factors was probably not primarily by mechanism *a* (disintegration of dead cells). In these particular experiments, the longer irradiation periods corresponded, also, to longer extraction periods. Hence either mechanism *b* or mechanism *c* could account for the results.

2. *Relation of yield to number of cells killed.* In a series of seven experiments, yeast suspended in isotonic salt solution was irradiated with full ultraviolet at

intensity levels which varied from experiment to experiment. Aliquots were removed at intervals for the determination of percentages of dead cells by methylene blue staining, and of the potencies of the intercellular fluids. Two examples, which are representative, are summarized in Fig. 2. The maximum staining referred to in each instance was between 97 and 100 %.

It will be noted from these examples that from 15 to 20 % of the maximum potency was attained by the time 2 % of the cells were killed, and roughly one-third of the maximum potency by the time 10 % of the cells were killed. This rather rapid rise in potency during the early periods of irradiation, before appreciable killing took place, seems unattributable to mechanism *a* and seems more easily explained by mechanism *b* or *c*.

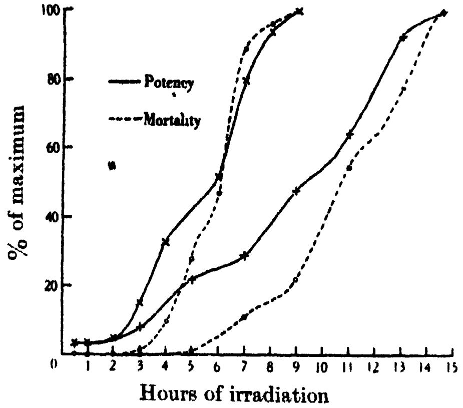


Fig. 2. Relation of yield of proliferation-promoting factors to mortality.

3. *Effect of injuring cells in various suspension media.* Cells suspended in distilled water or isotonic salt solution have no source of building materials for the synthesis of proliferation-promoting factors other than their own protoplasm. Consequently, if mechanism *c* (production of active materials by living, injured cells) accounts in part for the phenomena observed, one might expect to obtain greater yields of active materials from cells suspended in media containing available sources of nitrogen, phosphorus, carbohydrate etc.

Yeast was suspended at a concentration of 100 g. (wet weight) per l. in the following media: distilled water, isotonic NaCl, Reader's medium[1] [Reader, 1927], 95 % alcohol, and Ringer's glucose phosphate solution.[2] These suspensions were irradiated simultaneously in quartz test tubes with full ultraviolet at a distance of 15 cm. from a quartz mercury arc until 100 % of the cells in the salt solution stained when tested with methylene blue. Under these conditions, practically complete killing was obtained in all suspensions. Similar suspensions were kept standing without irradiation during the same interval as controls. All suspensions were then filtered through Berkefeld filters, and the cell-free fluids were taken to dryness. The dried products were dissolved in distilled water to one-fifth their original volume, taking care to suspend all the material. These solutions were assayed in the usual way. Table 1 shows the results.

---

[1] The composition of Reader's medium per 100 ml. is as follows: $(NH_4)_2SO_4$, 0·3 g.; $MgSO_4$, 7HO, 0·07 g.; KH_2PO_4, 0·10 g.; K_2HPO_4, 0·016 g.; NaCl, 0·05 g.; $Ca(NO_3)_2$, 0·04 g.; glucose, 0·5 g.

[2] The composition per 100 ml. was as follows: NaCl, 0·09 g.; $CaCl_2$, 0·024 g.; KCl, 0·001 g.; $Na_2H_2P_2O_7$, $6H_2O$, 0·006 g.; $NaHPO_3$, $3H_2O$, 0·205 g.; glucose, 0·02 g.

It will be noted that the most highly potent irradiation product and the least potent non-irradiated extract were those from cells suspended in Reader's medium. In the case of alcohol, the combined lethal effects of alcohol and irradiation resulted in lower potency per ml. of filtrate than extraction with alcohol alone. Since alcohol is an effective solvent for extracting growth-promoters of the bios type [e.g. Narayanan, 1930], it is not surprising that the alcohol preparations showed high activity.

All cells were killed in the various irradiated suspensions. Hence it is difficult to attribute the observed results to mechanism *a* alone. If increased permeability accounted entirely for the difference in potency of the irradiated and non-irradiated extracts, why was the difference greatest in the case of Reader's medium? If, on the other hand, mechanism *c* played a part in the phenomenon, the increased effect in Reader's medium is understandable because of the carbohydrate, nitrogen, phosphorus etc. supplied by it to the cells.

Table 1. *Effect of the suspension medium on the yield of proliferation-promoting factors from injured cells*

	Potency G.U. per ml.	
Suspension medium	Not irrad.	Irrad.
Distilled water	2·95	11·8
Isotonic NaCl	2·17	9·52
95% alcohol	13·3	10·9
Reader's medium	0·77	15·4
Ringer's G.P. solution	2·67	14·3

4. *Effects of CO_2 and O_2.* Because of the fermentation-stimulating effect of filtrates from irradiated as compared with non-irradiated cells [Cook & Loofbourow, unpublished], it was believed of interest to determine the effects of CO_2 and O_2 on the production of proliferation-promoting factors by yeast [Loofbourow & Dwyer, 1939]. In six experiments, yeast, at a concentration (wet weight) of 100 or 200 g./l., was suspended in isotonic NaCl. The suspensions were divided into three portions of 75 ml. each. Through one portion, CO_2 was bubbled continuously; through another portion O_2. The third portion had nothing bubbled through it. After 24 or 48 hr. of such treatment, the suspensions were filtered through Berkefeld filters, and the cell-free fluids were dried and assayed.

In these experiments, the cells were not killed. The maximum staining observed at the ends of the periods was of the order of 2%. The greatest potencies were in the filtrates from yeast exposed to CO_2, the next in the control filtrates, and the least in the filtrates from the cells exposed to O_2. Table 2 is from a representative experiment. Since no appreciable killing took place in these experiments, mechanism *a* appears improbable, and mechanisms *b* and *c* more likely. The effect of CO_2 on membrane permeability is well known. Hence one cannot say from these experiments whether *b* or *c* or both were involved.

Table 2. *Effect of CO_2 on the production of proliferation-promoting factors by yeast*

Sample	Cell-free filtrates Potency G.U. per ml.
CO_2	6·6
Untreated	3·1
O_2	1·5

The possible effect of accumulated CO_2 in leading to production of proliferation-promoting factors must evidently be considered when investigating the action of various injuring agents in inducing the production of such factors.

5. *Comparison of yields from cells fragmented by grinding before and after irradiation with lethal ultraviolet.* The following experiments were suggested by Prof. A. J. Carlson of the University of Chicago. They are based on the consideration that if active factors are produced by living cells as a response to injury (mechanism *c*), the yield should be less from cells ground until they are fragmented and then irradiated dead than from cells irradiated while alive and then ground. In both instances the end-products would, theoretically, be the total water-soluble cell contents freed from the cell—hence no questions of membrane permeability or efficiency of extraction should be involved.

Two equal weights of moist yeast (10–30 g.) were taken. One of these was ground with sand in a mortar until the cells appeared to be well fragmented. Both portions were suspended in isotonic NaCl at a concentration of 100 g./l. and irradiated in quartz test tubes with full ultraviolet until 50–100 % (the percentage varied from experiment to experiment) of the cells in the unground suspension were killed. The suspensions were centrifuged, and the supernatant fluids decanted. The unground cells were then ground in a mortar, after which both residues were resuspended in their respective fluids. The suspensions were later filtered through Berkefeld filters, and the cell-free filtrates dried and assayed. The purposes of filtration were to remove insoluble residues which might interfere with the optical estimation of yeast growth and to render the solutions sterile.

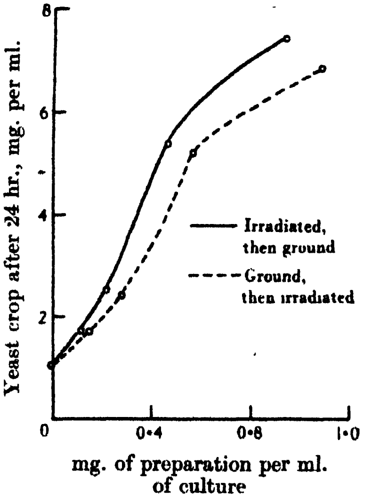


Fig. 3. Effect of killing cells by grinding before irradiation.

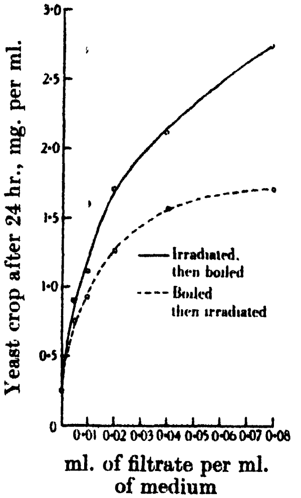


Fig. 4. Effect of killing cells by boiling before irradiation.

The products from cells irradiated while alive were slightly more active, both on a weight basis and equivalent filtrate volume basis, but the differences were small. Fig. 3 is a representative assay. The weight yields averaged from 20 to 30 mg./ml. in various experiments (compared with 2–5 mg./ml. in techniques in which unfragmented cells were used). Considerable difficulty was encountered in fragmenting all the cells by the method used.

The higher yields in the case of cells irradiated while alive seem to indicate that mechanism *c* was involved in the production of active factors.

6. *Comparison of yields from cells killed by boiling before irradiation and boiled after irradiation.* As an alternative to the above procedure, yields from cells killed by boiling were compared with yields from cells irradiated while alive. Yeast was suspended in isotonic NaCl, at 100 g./l. and divided into two aliquots. One of these was boiled for 3 min. to kill the cells. Both were then irradiated in quartz test tubes with full ultraviolet until all cells in the unboiled suspension were killed (methylene blue test). The irradiation time in the experiment illustrated in Fig. 4 was 4·25 hr. The distance from the quartz mercury arc was 15 cm. Following irradiation, the unboiled suspension was boiled for 3 min. Both suspensions were filtered through Berkefeld filters, and their filtrates were dried and assayed.

The suspensions irradiated before boiling gave more potent filtrates. This may be interpreted as indicating that proliferation-promoting factors are produced in living cells during injury (mechanism *c*) or that previous boiling interferes with extraction and results in lower yields from suspensions boiled before irradiation. The later interpretation does not seem to explain the phenomenon completely, however, because the filtrates from the suspensions irradiated while alive were more potent per unit weight as well as per unit volume.

DISCUSSION

The question as to whether the increased proliferation-promoting effect of intercellular fluids from injured cells may be attributed in part to factors produced by living cells as a specific response to injury is an extremely difficult one to answer with finality. Certainly, there exist in yeast cells many substances stimulating yeast growth, such as amino-acids, pantothenic acid, vitamin B_1, vitamin B_6, biotin etc., as has already been pointed out repeatedly in connexion with this problem [Cook *et al.* 1940, Davidson, 1940; Loofbourow & Morgan, 1940]. When cells are injured or killed, the release into the intercellular fluids of such substances present in the uninjured cell might be expected to complicate any attempt to determine the presence of factors specifically produced as a result of injury. Nevertheless, the greater potencies of preparations from cells injured slowly rather than killed rapidly, the rapid increase in such factors in the intercellular fluids before appreciable killing, the appearance of such factors in suspensions of living cells subjected to CO_2, and finally, the greater yields from cells fragmented or killed by boiling after rather than before prolonged injury all suggest that living, injured cells do produce proliferation-promoting factors (intercellular wound hormones) while undergoing injury.

Various evidence that proliferation-promoting intercellular hormones from injured cells are related to nucleic acids has been presented [Loofbourow, Cook & Stimson, 1938; Cook *et al.* 1939]. The results of Davidson [1940] in which assays of extracts from irradiated cells showed markedly higher nucleoside- and free purine-nitrogen than extracts from non-irradiated or autoclaved cells suggest this same conclusion. It is difficult to explain Davidson's results as entirely due to increased membrane permeability following irradiation or to the release of dead cell disintegration products as a result of irradiation, for the nucleoside- and purine-nitrogen was three times as great in his filtrates from irradiated as in his filtrates from autoclaved cells, whereas the dry weights of the materials obtained were roughly 3 to 2 in favour of the autoclaved cell extracts. These results seem to us an argument for the production of nucleic-acid-like materials as a specific response to injury.

The suggestion is frequently made that our intercellular wound hormones may be synthesized by a specific photochemical process in the cell. The fact that the active materials are obtained from cells injured in other ways and the indications that they are produced in living cells rather than by an effect of ultraviolet on the dead constituents of protoplasm are in our opinion arguments against this possibility.

SUMMARY

In order to determine whether the increase in the proliferation-promoting effect of intercellular fluids following cell injury is due to (*a*) release of dead cell disintegration products, (*b*) increased permeability of the cell membrane or (*c*) formation of proliferation-promoting intercellular hormones by cells as a specific response to injury, the following types of experiments were tried.

(1) A comparison was made of the effects of rapid killing and of slow injury. The yield of proliferation-promoting intercellular hormones was, within limits, closely proportional to the time required to kill the cells.

(2) The yield of active factors was compared with the number of cells killed and found to increase rapidly before appreciable killing took place.

(3) The yields from cells injured in various media were compared and found to be highest when the medium contained carbohydrate, nitrogenous substances, phosphoric acid etc.

(4) CO_2 was found to increase the yield of active factors in the intercellular fluids without killing the cells.

(5) Cells exposed to lethal ultraviolet before grinding yielded more potent preparations than cells irradiated after grinding.

(6) Cells irradiated with lethal ultraviolet after they were killed by boiling yielded less potent preparations than those irradiated before boiling.

The results suggest that in addition to possible factors arising in the intercellular fluids as a result of mechanisms *a* and *b*, at least part of the potency must be attributable to factors arising from mechanism *c*.

REFERENCES

Arey (1936). *Physiol. Rev.* **16**, 327.
Carrel (1930). *Proc. Inst. Med., Chicago*, **8**, 62.
Cook, Hart & Stimson (1940). *Biochem. J.* **34**, 1580.
—— Loofbourow & Stimson (1939). *Atti* X^{o} *Cong. Intern. Chim.* **5**, 26.
Davidson (1940). *Biochem. J.* **34**, 1537.
Fardon, Norris, Loofbourow & Ruddy (1937). *Nature, Lond.*, **139**, 589.
Loofbourow (1941). *Biol. Rev.* (in the Press).
—— Cook, Dwyer, & Hart (1939). *Nature, Lond.*, **144**, 553.
—— —— & Stimson (1938). *Nature, Lond.*, **142**, 573.
—— Cueto & Lane (1939). *Arch. exp. Zellforsch.* **22**, 607.
—— & Dwyer (1938, 1). *Science*, **88**, 191.
—— —— (1938, 2). *Studies Inst. Divi Thomae*, **2**, 129.
—— —— (1939). *Nature, Lond.*, **143**, 725.
—— —— & Morgan (1938). *Studies Inst. Divi Thomae*, **2**, 137.
—— —— & Lane (1940). *Biochem. J.* **34**, 432.
—— & Morgan (1938). *Studies Inst. Divi Thomae*, **2**, 137.
—— —— (1940). *J. Bact.* **39**, 437.
Menkin (1940). Dynamics of Inflammation. New York: The Macmillan Co.
Narayanan (1930). *Biochem. J.* **24**, 6.
Norris & Ruddy (1937). *Studies Inst. Divi Thomae*, **1**, 53.
Reader (1927). *Biochem. J.* **21**, 901.
Richards (1932). *Arch. Protistenk*, **78**, 263.
Sperti, Loofbourow & Dwyer (1937, 1). *Nature, Lond.*, **140**, 643.
—— —— —— (1937, 2). *Studies Inst. Divi Thomae*, **1**, 163.
—— —— & Lane (1937, 3). *Science*, **86**, 611.
Wiesmer (1892). Elementarstruktur. Vienna.

64. ENZYMIC DECOMPOSITION OF AMINO-SUGARS

By CECILIA LUTWAK-MANN[1]

From the Biochemical Laboratory, Cambridge

(*Received 31 May 1941*)

CONTINUING the study of the biochemical properties of cartilage and of other tissues which make up the joint [Lutwak-Mann, 1940, 1], experiments were carried out with chondroitin and mucoitin sulphuric acids, both of which occur in joint tissues (cartilage, tendons, mucous membranes). However, preliminary experiments have shown that comparatively little progress could be made in this field unless more information was available about the components of these complex substances and in particular, about the amino-sugars. In this paper results are given which describe the behaviour of amino-sugars under the influence of enzymes in animal tissues, yeast and bacteria. A preliminary account was given to the Biochemical Society [Lutwak-Mann, 1940, 2].

Materials

The following substances were used as substrates: glucosamine HCl, *N*-acetylglucosamine made according to White [1940], chondrosamine (galactosamine) HCl obtained by acid hydrolysis of chondroitin sulphuric acid, glucosaminic acid prepared from glucosamine by oxidation with HgO [Pringsheim & Ruschmann, 1915], chondroitin sulphuric acid from cartilage and from tendons made according to the method of either Levene & La Forge [1913] or Meyer & Smyth [1937].

For most experiments the animal tissues were used in the form of tissue slices but in some instances the tissues were minced and reduced to a pulp. There were also a few experiments where amino-sugars were fed or injected into animals (rats).

Yeast and bacteria were used as washed suspensions and only occasionally in the form of cell-free extracts.

Methods

Several methods were used to study the changes which amino-sugars undergo in the presence of various tissues and cells. Changes in the gas uptake or output were measured manometrically, using Barcroft differential manometers with side bulbs and gas outlets. In addition, the rate of decomposition of amino-sugars was estimated colorimetrically by the method of Elson & Morgan [1933]. The reducing sugar values were determined by the method of Hagedorn & Jensen [1923]. In all experiments the deamination of the amino-sugars was also studied and the NH_3-N was determined using the Parnas & Heller apparatus [1924]. The content of amino-N was occasionally estimated in the Van Slyke apparatus. It may be stated here that glucosamine HCl, chondrosamine HCl and *N*-acetylglucosamine were tested and found to yield no NH_3 in the presence of alkalis like borax, MgO, or $NaHCO_3$, which were used for the distillation of NH_3.

For the most part, rat and rabbit tissues were used, but some experiments were done with tissues of other animals (pig, horse, cat, guinea-pig).

---

[1] Fellow of Newnham College, Cambridge.

EXPERIMENTAL

A. *Behaviour of amino-sugars under the influence of animal tissues.* Increases in O_2 uptake and formation of NH_3 were invariably observed when amino-sugars were added to tissue slices from the following organs: kidney (cortex), testis, brain (cortex). Ovaries, retina and lung also showed certain rather small effects. On the other hand, negative results were obtained with liver, muscle (skeletal and heart), mucous membranes (intestine, joint), cartilage, spleen, red cells, plasma and synovial fluid. In Table 1 experiments are given which show the activities of a number of tissues towards glucosamine HCl, chondrosamine HCl and *N*-acetylglucosamine.

Table 1. *Oxidation and deamination of amino-sugars by animal tissues*

120 mg. wet wt. tissue slices. Ringer-phosphate *p*H 7·6; 10 mg. substrate. Barcroft manometers filled with O_2; KOH in inner tube.

			Glucosamine HCl		Chondrosamine HCl		*N*-acetyl-glucosamine	
Tissue	Exp.	Time hr.	µl. O_2*	µg. NH_3-N	µl. O_2	µg. NH_3-N	µl. O_2	µg. NH_3-N
Testis (rat)	1	½	72	22	60	10	80	8
	2	1	190	38	88	19	110	12
	3	1	150	32	100	22	170	20
		2	240	48	170	30	208	28
	4	4	—	128	—	42	—	48
Kidney cortex (rabbit)	1	⅓	40	16	30	3	36	Trace
	2	1	160	28	80	12	120	12
		3	240	68	160	28	220	28
		4	410	140	270	48	—	46
Brain (rat)	1	1	68	20	42	13	18	Trace
		2	108	30	96	26	42	12
Brain (rabbit)	2	4	—	78	—	—	—	—
Retina (pig)		2	190	28	78	16	190	4
Ovaries (cat)		2	—	32	—	—	—	16
Lung (rabbit)		2	—	29	—	12	—	12

* Values for O_2-uptake in this and all other tables refer to the 'extra'-O_2 uptake due to the substrate.

It can be seen from Table 1 that there are differences in the behaviour of the various tissues towards the three amino-sugars. In kidney and in testis there is comparatively little difference in the O_2 uptakes due to glucosamine HCl and *N*-acetylglucosamine. However, the deamination of the latter compound is slower than that of glucosamine. The enzyme system present in brain cortex shows little activity towards *N*-acetylglucosamine. It may be mentioned here that when experiments were carried out with mixtures of glucosamine and acetylglucosamine, the rates of deamination of both substances remained unaltered. The decomposition of chondrosamine HCl in all tissues examined is less rapid than that of glucosamine.

Before describing in detail the properties of the enzyme system involved in the breakdown of amino-sugars, it should be stated that in animal tissues their deamination was found to occur only aerobically. In the absence of O_2 the amino-sugars appeared to be stable in the presence of animal tissues; yeast and bacteria, on the other hand, were found to be active anaerobically as well (*v. inf.*). A distinct fall in *p*H due to acid production accompanied the O_2 uptake; no free CO_2 appeared to be formed in the course of the reaction.

Optimum p*H*. The breakdown of amino-sugars depends greatly on the *p*H which affects the oxidation and the deamination equally, so that it was impossible to separate the two processes by altering the acidity. The optimum *p*H was found to lie between 7·4 and 7·8. At *p*H 6·5 the reaction was very slow and below *p*H 6·0 it did not occur. Besides the phosphate buffer, *p*H 7·6, which was used throughout most of this work, borate, veronal and bicarbonate + CO_2 buffers were also tried. In all these buffers the reaction was slightly slower than in phosphate. In the presence of bicarbonate alone, both the O_2 uptake and the NH_3 production, though very rapid at the beginning, fell off nearly completely in the course of 30 min.

Comparison between the rate of O_2 uptake; NH_3 production and glucosamine disappearance. The experiments with animal tissues indicated that the O_2 uptake preceded the NH_3 formation, and similar results were obtained with yeast and bacteria, as will be seen later.

The results in Table 1 show that considerable amounts of O_2 were taken up in the course of amino-sugar breakdown, before any appreciable quantities of NH_3 could be detected. More detailed information can be gathered from the data in Tables 2–4. The experiments in Table 2 show that glucosamine added to tissue slices under aerobic conditions disappears fairly rapidly during the first 60–90 min., as shown both by the colorimetric and reduction values. The NH_3 liberation is very slow during this initial phase. In the later stages of the reaction the rate of amino-sugar disappearance falls considerably while the NH_3 production increases. However, even after prolonged periods of incubation, when more than ¾ of the substrate added has disappeared, the NH_3 values correspond only to about ½–⅔ of the amino-sugar used up during this period. In Table 3, in addition to the amounts of glucosamine used up and the NH_3 liberated, data are also given for the amount of O_2 taken up, while Table 4 illustrates the effect of *p*H on the course of glucosamine decomposition.

Table 2. *Rate of glucosamine disappearance and NH_3 formation*

Tissue	Glucosamine HCl added	Aerobic incubation time hr.	mg. glucosamine HCl found		μg. NH_3-N formed
			By determination of reducing sugar	By colorimetric method	
Testis (rat)	4·8 mg. (≡312 μg. NH_2-N)	2½	2·5	3·0	62
		3½	2·0	1·7	85
Testis (rat)	3·0 mg. (≡195 μg. NH_2-N)	2	1·7	—	70
		3	1·38	—	85
Kidney (rabbit)	4·14 mg. (≡269 μg. NH_2-N)	1½	—	2 5	55
		3	—	1·8	100
Kidney (rabbit)	3·8 mg. (≡247 μg. NH_2-N)	¾	2·4	—	16
		1½	1·4	—	56
		3½	1·1	—	88
Kidney (rabbit)	4·0 mg. (≡260 μg. NH_2-N)	½	—	3·1	24
		2	—	1·9	70
		3½	—	1·42	104

These experiments suggested the possibility of an oxidation product being first formed from the amino-sugar and then undergoing deamination. The decrease in *p*H which, as was mentioned previously, accompanied the O_2 uptake made it probable that this intermediary oxidation product would be an acid. It was thought that glucosaminic acid might be the substance formed under

Table 3. *O_2 uptake, glucosamine disappearance and NH_3 production*

Tissue	Glucosamine HCl added	Aerobic incubation time hr.	O_2-uptake due to glucosamine μl. O_2	mg. glucosamine HCl found	μg. NH_3-N formed
Testis (rat)	2 mg. ($\equiv$130 μg. NH_2-N)	1¼	140	0·78	40
Brain (rabbit)	2 mg. ($\equiv$130 μg. NH_2-N)	1½	180	0·67	28

Table 4. *Effect of* p*H on decomposition of glucosamine*

Testis (rat). Added 5 mg. glucosamine HCl ($\equiv$358 μg. NH_2-N). Aerobic incubation.

Time (hr.)	*p*H	mg. glucosamine-HCl found		μg. NH_3-N formed
		By determination of reducing sugar	By colorimetric glucosamine estimation	
1½	7·6	3·7	3·3	55
	6·6	4·1	3·7	29
3½	7·6	2·5	2·2	130
	6·6	3·5	3·3	46

these circumstances. To test this possibility glucosaminic acid was prepared from glucosamine, but when tested with animal tissues it was found to be completely stable, both with regard to NH_3 formation and to O_2-uptake. The addition of glucosaminic acid to any of the amino-sugars tested in no way interfered with their usual course of breakdown. These results seemed to disprove the assumption that glucosaminic acid was the product responsible for the NH_3 formation from amino-sugars; other experiments which will be referred to below make its formation unlikely in the course of amino-sugar decomposition by the enzyme system described in this paper.

Effect of various substances on the oxidation and deamination of amino-sugars. It was stated before that, except in brain cortex, there is little difference in the rate of O_2-uptake due to glucosamine and *N*-acetylglucosamine, but that the deamination of the acetylated compound is generally very slow. It was thought therefore that this difference in the rates of deamination of the free and the acetylated glucosamine, could be utilized to prove the enzymic conversion of glucosamine into an acetyl (or other) derivative.

With this possibility in mind, the rate of NH_3 formation from glucosamine was examined in the presence of several substances which were considered likely to yield derivatives able to block the NH_2 group. Table 5 shows the effect of several organic acids, aldehydes, alcohols and sugars on the deamination. It was found that some of the substances used decreased not only the deamination of the amino-sugar but also the 'blank' NH_3 formation, which means that they have an inhibitory effect upon deaminases generally.

Among the acids tested, oxaloacetic acid showed a fairly strong inhibition of the NH_3 production from glucosamine, especially if used in high concentration. On the other hand, acetic, lactic and pyruvic acids caused not more than *c.* 10 % decrease. Butyric and β-hydroxybutyric acids showed a somewhat more marked effect, while oxalic acid had no influence.

The addition of acetaldehyde and methylglyoxal depressed the deamination to a moderate degree, but with *dl*-glyceraldehyde a much more pronounced effect was obtained, particularly if kidney cortex slices were used as the source of enzyme (Table 6).

Table 5. *Effects of various substances on the deamination of glucosamine*

μg. NH_3-N formed

			No additions		With additions		
Tissue	Exp.	Time hr.	Tissue alone	Tissue + glucosamine	Tissue alone		Tissue + glucosamine
Kidney	1	2	28	80	22	Acetate *M*/10	70
	2	2½	28	100	28	Butyrate *M*/20	60
		2½	28	100	28	β-Hydroxybutyrate *M*/20	85
	3	3	36	130	35	Oxaloacetate *M*/40	62
		3	36	130	28	Oxaloacetate *M*/15	32
		3	36	130	32	Oxalate *M*/20	110
Testis	1	1	15	65	13	Pyruvate *M*/20	60
		1	15	65	12	Lactate *M*/20	52
Brain	1	2	30	56	40	Formate *M*/20	60
		2	30	56	30	Citrate *M*/20	48
Kidney	1	2	26	90	20	Acetaldehyde *M*/20	78
		2	26	90	18	Methylglyoxal *M*/30	50
		2	26	90	15	Glyceraldehyde *M*/20	30
Kidney	1	3	38	122	20	Glycerol *M*/40	77
	2	2	22	90	22	Menthol *M*/50	78
		2	22	90	26	Borneol *M*/50	90
Testis	1	3	30	112	20	Glucose *M*/20	42
	2	3	37	96	8	Glucose *M*/5	20
Kidney	1	1½	22	60	18	Glucose *M*/5	30
	2	3	32	125	20	Glucose *M*/20	56
		3	32	125	26	Cori ester *M*/10	70
		3	32	125	27	Fructose *M*/10	73
		3	32	125	36	Galactose *M*/10	120
		3	32	125	36	Sucrose *M*/8	140
Brain	1	2	15	49	4	Glucose *M*/8	22
	2	2½	40	80	41	Gluconate *M*/8	76
		2½	40	80	44	Glucurone *M*/10	78

Table 6. *Effect of glyceraldehyde on glucosamine deamination in various tissues*

μg. NH_3-N formed

			No additions		With glyceraldehyde		
Tissue	Exp.	Time hr.	Tissue alone	Tissue + glucosamine	Tissue alone		Tissue + glucosamine
Kidney	1	2	22	84	13	Glyceraldehyde *M*/50	37
	2	1½	20	66	18	Glyceraldehyde *M*/100	30
			20	66	16	Glyceraldehyde *M*/30	22
Testis	1	2½	36	85	27	Glyceraldehyde *M*/50	48
	2	1½	15	47	10	Glyceraldehyde *M*/60	31
Brain	1	1½	26	40	23	Glyceraldehyde *M*/50	32

When various sugars were examined for their ability to influence the deamination of glucosamine, it was found that glucose alone among several sugars tested strongly interfered with the amino-sugar deaminase. It was possible, by using higher concentrations of glucose, to prevent the deamination of glucosamine nearly completely (Tables 5 and 7). Except for glucose-1-phosphoric acid (Cori ester), none of the other sugars could be compared with glucose in its effect upon the deaminating enzyme.

It seemed desirable to investigate more closely the behaviour of amino-sugars in the presence of glyceraldehyde and glucose, not only in connexion with their effects upon the deamination but also with regard to the O_2-uptake. The colorimetric estimation of glucosamine disappearance was, however, impossible in the presence of glyceraldehyde, owing to the fact that the presence of this aldehyde prevented the formation of colour in the method of Elson & Morgan. Similarly, it was impracticable to attempt to assess the reducing sugar values for the amino-sugar in the presence of glucose; in the colorimetric method glucose caused no interference.

It can be seen from Table 7 that glucose had a more pronounced effect on glucosamine deamination than glyceraldehyde but that glyceraldehyde inhibited the O_2-uptake more than glucose. The same table contains also data obtained with lactate and pyruvate, which had little effect upon either the oxidation or the deamination of glucosamine.

Table 7. *Effects of glucose, glyceraldehyde, lactate and pyruvate on the O_2 uptake and NH_3 formation*

Tissue	Time		μl. O_2 taken up	μg. NH_3-N formed
Testis	2 hr. 30 min.	No addition	290	78
		Glucose *M*/15	205	29
Kidney	1 hr. 30 min.	No addition	172	48
		Glucose *M*/12	119	12
Testis	1 hr. 30 min.	No addition	210	42
		Glyceraldehyde *M*/60	38	21
Testis	50 min.	No addition	120	30
		Lactate *M*/20	90	28
		Pyruvate *M*/20	148	33

In some of these experiments the content of amino-N was estimated in the samples at the beginning and end of the experiment, both without and with the addition of substances like glyceraldehyde, glucose, oxaloacetate, lactate and pyruvate. But the results obtained completely failed to produce any evidence that the glucosamine amino-group was blocked, as might have been expected if acetylation or a similar process had taken place during the incubation of glucosamine with these substances.

To ascertain whether the effect of glucose on the glucosamine deamination was due to glucose itself or to its breakdown products, experiments were carried out in which the tissue slices were incubated for a time with glucose, either in air or anaerobically, before the amino-sugar was added. From the data in Table 8 it appears that the presence of glucose oxidation products had practically the same effect on the deamination of glucosamine as glucose alone; there was, however, a small increase in the amount of O_2 used up. When the tissue was kept with glucose in N_2, before the addition of glucosamine, the results did not differ

Table 8. *Effect of glucose and of its products on glucosamine oxidation and deamination*

Tissue slices (testis) incubated for 1 hr. with glucose in O_2 (*c*) or in N_2 (*d*), before addition of glucosamine.

	μl. O_2/1 hr.		μg. NH_3-N/1 hr.	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
(*a*) Glucosamine	156	160	52	61
(*b*) +glucose (no incub.)	100	118	20	13
(*c*) +glucose (O_2 incub.)	180	195	18	10
(*d*) +glucose (N_2 incub.)	94	100	20	4

from those obtained when glucose and glucosamine were added simultaneously to the tissue slices.

Interesting results were obtained when glucose and galactose were compared in their effects on the oxidation and deamination of chondrosamine. The experiment in Table 9 shows that while galactose had comparatively little effect on the deamination of this amino-sugar, it interfered more with its oxidation than glucose.

Table 9. *Effects of glucose and galactose on chondrosamine breakdown*

Tissue		μl. O_2 taken up in 90 min.	μg. NH_3-N formed in 90 min.
Kidney	Chondrosamine	280	52
	+glucose *M*/10	210	20
	+galactose *M*/10	170	40

Poisons and inhibitors. With the intention of obtaining some indication as to the type of reaction involved in the enzymic decomposition of amino-sugars, several substances were tested with regard to their effects on the enzyme system and the results are collected in Table 10. The experiments in this table were carried out using kidney cortex slices, but analogous results were obtained with other active tissues.

Table 10. *Effects of poisons and inhibitors on glucosamine oxidation and deamination*

	μl. O_2 taken up/100 min.	μg. NH_3-N formed/100 min.
Without additions	230	70
With		
Iodoacetate *M*/100	0	0
Fluoride *M*/50	156	44
Arsenate *M*/75	130	33
Cyanide *M*/250	56	52
Cyanide *M*/75	32	29
Azide *M*/75	190	58
Malonate *M*/100	200	67
Cinchophen *M*/10	0	0
Salicylate *M*/50	180	43
Toluene	0	0
Sulphapyridine	248	75
Merthiolate 1 : 20000	0	0

It was interesting to discover that the reaction under investigation is extremely sensitive to iodoacetic acid (IA). Both oxidation and deamination of amino-sugars were abolished by this poison. Smaller effects were obtained with NaF and arsenate, while malonate and azide caused no inhibition at all. KCN largely prevented the oxidation, but had comparatively little effect on the NH_3 formation. Salicylic acid was found to be a mild inhibitor, but cinchophen (phenyl cinchoninic acid) suppressed completely the activity of the amino-sugar enzyme system. Organic solvents like alcohol, acetone, ether and toluene destroyed the enzyme, even in low concentrations. It was also inactivated in the presence of merthiolate. On the other hand, sulphanilamide and sulphapyridine had no influence on the reaction. On several occasions the tissues used for the experiments with amino-sugars, were removed from animals which had received sublethal doses of either sulphapyridine or sulphanilamide; yet the enzyme system responsible for the decomposition of amino-sugars remained entirely unaffected.

The effect of semicarbazide and dimedone upon the rate of glucosamine breakdown was also tested; no significant changes, however, were observed, as might have been the case if ketones or aldehydes had been produced in the course of the reaction. The lack of effect with these 'trapping' substances agreed well with the negative results obtained for bisulphite-binding substances (*vide infra*).

Tissues other than kidney, testis and brain. It was mentioned previously that a slight O_2 uptake and deamination of amino-sugars could be observed with ovaries, retina and lung tissue. Among other animal tissues and fluids which were tested in various ways and found to contain no enzymes capable of either the oxidation or deamination of amino-sugars, were liver, muscle, intestine, cartilage, blood (whole and haemolysed), serum and synovial fluid.

Particular attention was paid to liver as this tissue is well known to possess enzymes which might be related to the system dealing with amino-sugars, such as glucose oxidase and amino-acid oxidase. However, in all experiments with liver tissue, used either in the form of slices or pulp, neither oxidation nor deamination of amino-sugars took place. The absence of deaminase activity was not due to substitution of the amino-group, because it was found after prolonged incubation of glucosamine with liver that the amino-group remained free even when such substances as acetate, lactate, pyruvate etc., were added.

It may be of some interest to mention here that the addition of amino-sugars greatly interfered with the oxidation of glucose by liver slices (Table 11). A similar effect of amino-sugars upon glucose oxidation was also observed in testis slices; in this instance there was, of course, a mutual interference of glucose and amino-sugar as described on p. 615.

Table 11. *Effect of amino-sugars on glucose oxidation in liver*

	μl. O_2 taken up/70 min.
Glucose	205
+ glucosamine	100
+ chondrosamine	130
+ *N*-acetylglucosamine	84
Glucosamine	0
Chondrosamine	0

It appeared fairly probable from the results obtained with liver that this organ is devoid of enzymes acting on amino-sugars. It seemed, however, worth while to investigate whether the apparent stability of the amino-sugars in liver was not due to the presence of substances capable of suppressing their decomposition.

Liver slices were incubated aerobically for 1–2 hr. in Ringer-phosphate; the tissue was then removed and the liquid was heated for 5 min. to 80°. When this heat-inactivated fluid was added to kidney cortex slices, it considerably retarded the deamination of glucosamine. Similar effects were obtained by adding the extract from liver to brain or testis slices. If, however, the liver slices were incubated anaerobically instead of aerobically, the effect of the heat-inactivated extract was much less conspicuous. Further experiments of this kind showed that the liver factor responsible for the suppression of amino-sugar deamination, is dialysable and heat-stable and also that it is not formed aerobically at *p*H below 6·0 (Table 12). On the other hand, its formation in the liver was not prevented by KCN, phloridzin or semicarbazide. Heat-inactivated extracts from kidney or testis slices, or boiled yeast juice, failed to produce any marked effect on the NH_3 production from amino-sugars.

Table 12. *Effect of heat-inactivated liver extract on the deamination of glucosamine by kidney cortex*

	μg. NH_3-N produced/1 hr. 30 min.
Glucosamine	68
+ liver extract, aerob., pH = 7·4	22
+ liver extract, dialysed	50
+ liver extract, aerob., pH = 4·8	60
+ liver extract, anaerob., pH = 7·4	42

In experiments with muscle or mucous membranes, it was necessary to mince the tissues and to use them in the form of a pulp. As stated previously, negative results were obtained with these tissues, except for some very slight anaerobic oxidation of glucosamine by washed heart muscle mince in presence of cozymase and methylene blue (Table 15). In this connexion it should be stated that when active kidney cortex or testis slices were broken up by grinding in Ringer-phosphate and made into a thick pulp, their activity towards amino-sugars was considerably reduced (Table 13). It would seem therefore that the enzyme system dealing with amino-sugars depends largely upon the tissue structure being preserved intact. Completely negative results were obtained with a kidney cortex acetone powder preparation, although this preparation was very active towards d-alanine. Also a preparation of testicular diffusing factor (obtained through the kindness of Dr Bacharach) appeared devoid of any power to oxidize or to deaminate amino-sugars.

Table 13. *Decomposition of amino-sugars by tissue slices and by pulp*

Tissue	Preparation	Substrate	μl. O_2/2 hr.	μg. NH_3-N/2 hr.
Kidney cortex	Slices	Glucosamine	260	79
		N-acetylglucosamine	190	21
	Pulp	Glucosamine	56	38
		N-acetylglucosamine	Not measured	Trace
Testis	Slices	Glucosamine	190	88
		N-acetylglucosamine	142	34
	Pulp	Glucosamine	Very small	52
		N-acetylglucosamine	Very small	18

Attempts to identify the reaction products. The distinct fall in pH accompanying the breakdown of amino-sugars, made probable the formation of an acid in the course of the reaction. Estimations of lactic and acetic acids were negative. The presence of succinic acid was made improbable by experimental results described below.

Estimations of bisulphite-binding substances [Clift & Cook, 1932] gave negative results. No substances were formed which would yield derivatives with 2:4-dinitrophenylhydrazine.

A special series of experiments was carried out to investigate more thoroughly the formation of glucosaminic acid. It was mentioned already that glucosaminic acid was found to be remarkably stable in the presence of animal tissues and therefore it could hardly be regarded as the (hypothetical) oxidation product from glucosamine which undergoes deamination. Nevertheless, it seemed important to examine whether glucosaminic (or chondrosaminic) acid was formed from the amino-sugars under the experimental conditions described in this paper.

This was done in the following manner. It was found that glucosaminic acid yields CO_2 readily in the presence of chloramine-T at *p*H 4·6, in quantities roughly proportional to the amount of the acid, while glucosamine causes only a very slow gas production under similar conditions. The CO_2 evolution can easily be measured manometrically (Table 14).

Table 14. *Behaviour of amino-sugars and glucosaminic acid in the presence of chloramine-T*

	μl. CO_2/20 min., 38°, *p*H = 4·6
Glucosamine HCl, 2 mg.	15
Chondrosamine HCl, 2 mg.	12
Glucosaminic acid, 2 mg.	120
Glucosaminic acid, 4 mg.	195

The technique adopted for the estimation of glucosaminic acid largely followed the method described by Cohen [1940]. It was found necessary to remove NH_3 formed from glucosamine in the course of the enzymic reaction, in order to avoid N_2 formation by the chloramine-T treatment. This was conveniently accomplished by distillation in the Parnas-Heller apparatus.

Tissue slices (250 mg. wet wt., total volume of solution 3 ml.) were incubated with a known quantity of glucosamine in the usual manner. O_2 uptake and NH_3 formation were estimated and at various intervals the reaction was stopped by heating the sample to 90° for 3 min. After the removal of NH_3 and the precipitation of proteins by tungstic acid, the filtrate was condensed at 100° to half its original volume. The amount of fluid taken for the final chloramine-T experiment was chosen so as to contain between 2 and 4 mg. of glucosaminic acid. Numerous controls were carried out to ascertain that glucosaminic acid added to the tissue under the conditions referred to could be recovered without loss in spite of the prolonged preparatory manipulations.

However, from a series of experiments carried out with glucosamine and chondrosamine HCl, both alone and in the presence of glucose, it appeared certain that glucosaminic acid was not formed under these conditions, at least in any appreciable quantities. Similar negative results were also obtained using *B. coli* instead of the animal tissues.

The following experiments were carried out in yet another attempt to identify the reaction products formed during the breakdown of amino-sugars. Minced and thoroughly washed muscle pulp was prepared, such as is known to contain several dehydrogenases. Using this enzyme preparation, the effect was studied on the methylene blue reduction time of the addition of a heat-inactivated extract from tissue slices which had been allowed to act for a considerable time on amino-sugars. Table 15 gives the results of some of these Thunberg experiments. It can be seen that (*a*) glucosamine itself is very slowly oxidized by heart muscle enzymes in the presence of cozymase, and (*b*) in the tissue slices extract containing the reaction products from amino-sugars, there is a substance which in the presence of cozymase definitely accelerates the reduction of methylene blue. There are two substrates which do not require cozymase for their anaerobic oxidation with methylene blue by muscle enzymes: succinic and α-glycerophosphoric acids. These two can therefore be excluded as possible intermediary or end-products of amino-sugar breakdown. The formation of α-glycerophosphate was already unlikely, because separate experiments showed no evidence of phosphorylation being involved in the amino-sugar decomposition.

Table 15. *Effect of muscle dehydrogenases on the reaction products of amino-sugar breakdown*

0·3 g. muscle dehydrogenase preparation, 0·6 mg. cozymase, 1 ml. *M*/2000 M.B., *p*H = 7·4.

		Reduction time, min.
Heart muscle prep.		∞
	+ cozymase	95
	+ glucosamine	∞
	+ cozymase + glucosamine	28
	+ cozymase + chondrosamine	70
	+ extract from tissue slices	80
	+ cozymase + extract from slices	45
	+ extract from slices incubated with glucosamine	55
	+ cozymase + extract from slices incubated with glucosamine	12
Skeletal muscle prep.	+ cozymase	50
	+ cozymase + *N*-acetylglucosamine	40
	+ cozymase + extract from slices	38
	+ cozymase + extract from slices incubated with *N*-acetylglucosamine	14

Experiments with chondroitin sulphuric acid. Comparatively few experiments were carried out using this compound as substrate for tissue or bacterial enzymes; in fact, their variability was the main cause why they were abandoned for the time being and the research on amino-sugars was taken up instead.

Chondroitin Na-sulphate was added to tissue slices (kidney, testis) and incubated for 2, 4 and 6 hr. at 37°, at *p*H values varying between 6·0 and 8·0. Afterwards the supernatant fluid was examined for the presence of amino-sugars. Traces of amino-sugar could usually be detected but the amount varied considerably and it appeared probable that this was due to a further decomposition of the amino-sugar set free from chondroitin sulphuric acid by the tissue enzymes. This assumption was confirmed by the results of the work reported in this paper.

Attempts were also made to assess the rate of amino-sugar liberation during acid hydrolysis of chondroitin sulphuric acid. Use was made of the enzymes present in tissue slices to indicate the presence of amino-sugar. For this purpose, samples of chondroitin sulphuric acid were hydrolysed in sealed tubes at 100° in *N* HCl for 1, 2, 4 and 12 hr. They were carefully neutralized and freed from NH_3. They were then added to testis or kidney cortex slices and incubated aerobically in the usual manner. Production of NH_3 indicated the presence of amino-sugar, as chondroitin sulphuric acid itself gave rise to no NH_3 formation. In this way it was found that after 60 to 90 min. of acid hydrolysis chondroitin sulphuric acid yields a considerable amount of amino-sugar in a form in which it can be attacked by the tissue enzymes. An inconvenient complication of these experiments was caused by the rather high NaCl concentration of the hydrolysed and neutralized samples. It was ascertained in separate experiments that *N* NaCl or KCl completely inactivates the system acting on amino-sugars. It was therefore necessary to dilute the hydrolysed samples very considerably to diminish the salt concentration and thus only comparatively small fractions of the amino-sugar present were used for the final enzymic experiment.

Experiments with animals. One of the main obstacles, so far, to the study of amino-sugar metabolism is due to the difficulty of quantitative estimation of amino-sugar in urine. It appears probable that urea is the substance which interferes with the colorimetric estimation of amino-sugar in urine, as it was found that small amounts of urea give the same orange (instead of pink) colour with the Ehrlich reagent as is obtained with dilute urine.

As no successful and simple way could be found to apply the Elson & Morgan method to estimations in urine, it was decided to study instead the effect of amino-sugars on the NH_3-N content of the urine in animals which were known to possess organs able to deaminate amino-sugars. Glucosamine HCl (1 mg./g. body weight) in Ringer was injected subcutaneously into rats (180 g.) while Ringer alone was injected into control animals. The following method of urine collection was adopted. The food was removed early on the day of the experiment and urine was collected for some 10 hr. Then followed the injection and a further collection was made for 10–12 hr. Food was then restored and urine was collected for a further 4–6 hr. In all samples the total volume, *p*H and the NH_3-N were then estimated. Table 16 gives the results of some of these experiments. It can be seen that there were considerable variations both in the volume of urine excreted in a given time and in the NH_3-N content of the urine samples, even before the injection. In the samples following the injection there was a slight increase in the NH_3-N content of the urine in the animals which received glucosamine HCl, but this was very small indeed if compared with the NH_3-N content of the amino-sugar injected. There were no significant changes in the *p*H of the urine samples after the injection of the amino-sugar. It would seem that, at least in rats, the amino-sugars were prevented from complete deamination, although *in vitro*, in the presence of rat tissues like kidney or testis, they would have lost their amino-group in the course of prolonged incubation.

Table 16. *Effect of subcutaneous injection of glucosamine on the NH_3 content of urine in rat*

Urine sample	Volume (ml.)			*p*H			mg. NH_3-N in total urine		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Fasting animals:									
7 hr. before injection of saline	3	15	1	6	6·9	5·9	6·00	4·95	1·40
7 hr. before injection of glucosamine	7	18	1·3	5·9	6·6	6·0	6·16	9·00	0·80
Fasting animals:									
12 hr. after injection of saline	2·6	8	3	6	6·6	5·8	2·47	2·80	4·20
12 hr. after injection of glucosamine	3·4	6	9	5·9	6·5	6·2	3·40	6·00	7·20
Fed animals, 10 hr. after above collection:									
Saline-injected animals	11	8·5	—	7·3	7·5	—	2·53	3·65	—
Glucosamine-injected animals	16·5	6·5	—	7·0	6·9	—	2·55	3·30	—

It seems worth while to report briefly certain other experiments, which although interesting cannot at present be extended owing to the difficulties in obtaining large quantities of dried egg white. Male albino rats were kept on the 'egg white injury'-producing diet [György, 1939]. After a period of about 120 days they developed the typical skin and spectacled eye condition characteristic of egg white injury. Some of these animals were given 20 mg. of glucosamine HCl per day for a week, during which period they began to show a definite improvement such as growth of hair on the most affected skin areas as well as a distinct clearing up of the eye exudates. Their weight was maintained at a constant level. Chondrosamine HCl tested under similar conditions failed to produce any effect at all. However, after some 8 days of improvement due to glucosamine its effect faded and a relapse followed, which was not relieved by further doses of glucosamine HCl.

In a different series of experiments the effect of glucosamine feeding was investigated on B_2-deficient rats. Here however, no visible results of any kind were obtained. The tissues of both egg white-fed and B_2-deficient rats possessed the enzyme system concerned with the breakdown of amino-sugars.

B. *Yeast enzymes.* Several experiments were carried out using suspensions of baker's or brewer's yeast as the source of enzymes acting on amino-sugars. An enzyme system was found to exist in yeast which very slowly deaminated glucosamine and chondrosamine HCl in aerobic as well as in anaerobic conditions. The enzymes showed very little activity towards *N*-acetylglucosamine (Table 17). No free CO_2 appeared to be formed either in air or in N_2. As in animal tissues, the rate of oxidation exceeded the NH_3 formation; there was also the formation of acid reaction products which have not yet been identified.

Table 17. *Decomposition of amino-sugars by baker's yeast*

12 mg. yeast (dry wt.), 10 mg. substrate, $pH = 7{\cdot}6$.

	Oxygen uptake $\mu l.\ O_2/1$ hr.	Aerobic NH_3 formation $\mu g.\ NH_3\text{-}N/2\tfrac{1}{2}$ hr.	Anaerobic NH_3 formation $\mu g.\ NH_3\text{-}N/2\tfrac{1}{2}$ hr.
Glucosamine-HCl	120	60	28
Chondrosamine-HCl	72	34	11
N-acetylglucosamine	50	28	8
Glucose	1340	—	—

The yeast enzymes showed the same behaviour towards poisons, e.g. iodoacetate and KCN, as described previously in connexion with animal tissues. The specificity of the glucosamine deaminase in yeast was proved by the fact that while it was completely inhibited in the presence of iodoacetate, this poison did not affect the deamination by yeast of other substrates such as adenosine or adenylic acid.

Cell-free extracts from fresh baker's yeast, as well as Lebedew maceration juice from dried brewer's yeast, were found to be very weak in activity towards amino-sugars.

C. *Bacterial enzymes.* For most experiments *B. coli* was used in washed suspensions, but a few experiments were also done using suspensions of *Str. faecalis* and *Proteus vulgaris.*

B. coli was found to contain a powerful enzyme system which at pH 6·8–7·8 rapidly oxidized and deaminated glucosamine HCl and, a little more slowly, chondrosamine HCl and *N*-acetylglucosamine (Table 18).

Table 18. *Decomposition of amino-sugars by bacteria*

2 mg. suspension (dry wt.), 10 mg. substrate, $pH = 7{\cdot}6$.

	Glucosamine HCl	Chondrosamine HCl	*N*-acetyl-glucosamine
B. coli:			
$\mu l.\ O_2/40$ min.	180	110	130
$\mu g.\ NH_3\text{-}N/40$ min.: aerob.	98	62	80
$\mu g.\ NH_3\text{-}N/40$ min.: anaerob.	40	28	38
Str. faecalis:			
$\mu l.\ O_2/50$ min.	90	45	—
$\mu g.\ NH_3\text{-}N/50$ min.: aerob.	160	130	—
$\mu g.\ NH_3\text{-}N/50$ min.: anaerob.	140	41	—
Proteus vulgaris:			
$\mu l.\ O_2/60$ min.	68	90	—
$\mu g.\ NH_3\text{-}N/60$ min.: aerob.	18	29	—

The oxidation of amino-sugars was accompanied by acid formation. Also in anaerobic conditions *B. coli* deaminated the amino-sugars and produced acid reaction products from them. There was no CO_2 evolution in the course of either of these reactions.

Except for the ability to decompose the amino-sugars anaerobically, the bacterial enzyme system behaved in all respects like the animal system, i.e. the same substances were found to act as inhibitors and poisons in bacteria as described above for kidney slices or testis (Table 19).

Table 19. *Effects of glyceraldehyde, glucose, cyanide and iodoacetate on the decomposition of glucosamine by* B. coli

	μl. O_2/1 hr.	μg. NH_3-N/1 hr.: aerob.
Glucosamine	290	160
+ glyceraldehyde *M*/60	85	100
+ glucose *M*/20	188	36
+ cyanide *M*/100	60	130
+ iodoacetate *M*/150	0	0

The sequence of the processes in *B. coli* which cause the breakdown of amino-sugars appeared to be similar to that demonstrated for the animal enzymes, that is to say oxidation preceded deamination. By adding glucose to the bacterial suspension it was possible to diminish considerably the NH_3 liberation without much affecting the O_2 uptake. On the other hand, the addition of glucose to the culture medium of *B. coli* did not influence the amino-sugar deaminase in any marked way.

B. coli was also found to be capable of rapidly oxidizing the amino-sugars under anaerobic conditions in the presence of methylene blue as hydrogen acceptor. Table 20 A illustrates the results of experiments carried out with the Thunberg technique. It shows the effects of various poisons on the anaerobic oxidation, which are analogous with those obtained in aerobic conditions. Treatment of the bacterial suspension with toluene completely destroyed the enzyme activity.

Further experiments of this kind confirmed the assertion previously made that considerable amounts of the amino-sugar are oxidized before any large-scale deamination sets in. In the B section of Table 20 the results show that in the course of 16 min. roughly 3 mg. of methylene blue were reduced (or *c.* 2 mg. glucosamine HCl oxidized), but that the amount of NH_3 produced during this

Table 20. *Anaerobic oxidation of glucosamine by* B. coli

1 mg. bacterial suspension (dry wt.); 1 ml. *M*/2000 M.B.; *p*H = 7·4; total volume, 3 ml.

A.	Reduction time, min.
Blank	60
Glucosamine	4
Glucosamine, *p*H = 6·0	28
Glucosamine + iodoacetate *M*/100	49
+ fluoride *M*/70	37
+ cyanide *M*/100	5
+ toluene	65

B. 4 mg. bacterial suspension (dry wt.) in 3 ml. total volume.

	Reduction time, min.	μg. NH_3-N found
Blank + 2 mg. M.B.	70	24
Glucosamine + 1 mg. M.B.	4	40
+ 2 mg. M.B.	9	49
+ 3 mg. M.B.	16	80

period (0·056 mg. NH_3-N) corresponded to not more than half the amount of the substrate oxidized.

Various attempts were made to isolate the reaction products of glucosamine breakdown in *B. coli*, on lines similar to those adopted for animal tissues. It was established that there was no lactic or glucosaminic acid formation. The latter was found to be completely stable in the presence of bacteria.

Suspensions of *Str. faecalis* were found to be active towards the amino-sugars, but their oxidizing enzymes were less strongly developed than those of *B. coli*, while their deaminase was rather powerful, both in O_2 and in N_2.

Suspensions of *Proteus vulgaris* acted very slowly on glucosamine HCl both as regards O_2 uptake and deamination; they appeared, however, slightly more active towards chondrosamine HCl (Table 18):

DISCUSSION

From the experimental data presented above some information can be gathered about the enzyme system capable of amino-sugar decomposition. Its fairly wide distribution, as shown by the fact that it occurs in animal tissues, yeasts and bacteria, is of particular interest when one bears in mind that amino-sugars form an essential part of various complex polysaccharides which seem to play an important part in many physiological processes.

There is evidence from the experiments described that the enzyme system concerned in the decomposition of amino-sugars is strictly specific for these substances. Its non-identity with the glucose oxidase was easily proved by the results obtained with liver. This organ was incapable of attacking the amino-sugars although it rapidly oxidizes glucose. Moreover, amino-sugars were found to interfere with the glucose oxidase; this phenomenon may perhaps be regarded as an instance of competitive inhibition.

Furthermore, there appears to be no relationship between the amino-sugar enzyme and the amino-acid oxidase; this can be simply shown using acetone kidney cortex preparations, which, though strongly active towards *d*-alanine, fail completely to attack the amino-sugars. It was also found that while iodoacetic acid completely stopped the breakdown of glucosamine in kidney cortex slices, it had comparatively little effect on the decomposition of *l*-alanine. The amino-sugar enzyme is also distinct from the tyramine oxidase, as shown by experiments with intestinal mucosa which oxidizes and deaminates the amine but does not act on amino-sugars.

By applying iodoacetic acid to yeast it was possible to show that the glucosamine deaminase is distinct from the enzyme which deaminates adenine derivatives.

The enzyme system concerned with the amino-sugars seems to be composed of two closely linked enzymes of which one is responsible for the oxidation and the other for the deamination of the substrate. The evidence for this view is based on the observation of the behaviour of the enzyme system in the presence of various substances. For instance, by the addition of glyceraldehyde or KCN, one can largely depress the oxidation without much affecting the deamination; on the other hand, glucose or oxaloacetate considerably diminish the amount of NH_3 produced, but have less effect on the O_2 uptake. The fact that the enzyme largely depends on the integrity of cell structure, makes it difficult to resolve this system into its components. As the experiments with cell-free extracts from tissues or from other sources were few, it was not possible to determine whether this enzyme required any coenzyme or what the nature of this coenzyme might be.

Generally speaking, of the three amino-sugars tested, glucosamine is oxidized and deaminated more rapidly than either chondrosamine or acetylglucosamine; this difference in the rates of breakdown exhibited by the enzyme preparations tested may be due to a greater affinity for glucosamine than for galactosamine. The presence of the acetyl group in *N*-acetylglucosamine proves an obstacle to the deaminase but interferes comparatively little with the oxidation. In spite of many attempts to bring about an enzymic acetylation of glucosamine, no positive results have been achieved so far. But the difference in the rates of deamination of glucosamine and acetylglucosamine may be very useful as an indication of the blocking of the NH_2 group.

The nature of the reaction products in addition to NH_3 formed from amino-sugars has yet to be discovered. So far, it seems certain that there is no lactic, acetic or succinic acid among the breakdown products; substances of aldehyde or ketone character are also absent. With regard to the mechanism of the enzymic decomposition it appears probable that the amino-sugars undergo first an oxidation and that it is this oxidation product which subsequently gives off NH_3. The experimental results indicate that glucosaminic acid is not formed in the course of glucosamine oxidation and it can therefore be excluded as the reaction product which undergoes deamination. It was interesting to note the considerable stability of glucosaminic acid in the presence of enzymes from various sources.

From the experiments with the whole animal it follows that considerable amounts of amino-sugar can be injected without causing any marked change in the NH_3-N excretion. It is quite likely that in the intact animal the amino-sugars undergo enzymic processes different from those occurring in isolated tissues. The remarkable effect of glucosamine feeding in egg white injury, although transient and probably unspecific, shows that much more study is necessary in the field of amino-sugar metabolism, especially with regard to the nutritional requirements of the organism for these substances. At present no data seem to be available as to whether the amino-sugars can be formed within the organism, or whether they must be added with other exogenous foodstuffs.

Yeast and bacteria act on the amino-sugars both aerobically and anaerobically. The yeast enzyme is rather weak but the enzyme in *B. coli* decomposes amino-sugars very rapidly. The effects of various inhibitors on these enzymes are the same as in animal tissues.

Much attention has recently been given to amino-sugars in connexion with work on the diffusing factor and mucinase, which hydrolyse certain polysaccharides and liberate from them amino-sugars. It is unlikely that the sensitive enzyme system described in this work could play any part or be present in the preparations of testicular diffusing factor, which apart from being stable in the presence of antiseptics like merthiolate, are known to exert their maximum activity at a low *p*H, at which the enzyme causing the oxidative deamination of amino-sugars would no longer be active. In connexion with this it may be interesting to report that bee venom ('Lyovac', Mulford Laboratories) was found to oxidize and deaminate both glucosamine and chondrosamine HCl.

SUMMARY

An enzyme system which decomposes glucosamine HCl, *N*-acetylglucosamine and chondrosamine HCl, was found to exist in animal tissues, yeast and bacteria.

In animal tissues the addition of amino-sugars was found to cause an increase in the O_2 uptake, accompanied by acid formation and production of NH_3. No free CO_2 was formed in the course of the reaction. In yeast and bacteria, the

same processes occur but, in addition, the amino-sugars are attacked also in the absence of O_2.

The enzyme examined is specific for amino-sugars; it bears no relation to either the glucose oxidase or the amino-acid oxidase or the amine oxidase. Its activity was found to depend greatly on the cell structure being preserved. The optimum *p*H was found to lie between 7·4 and 7·8.

From quantitative estimations of the amino-sugar disappearing and the O_2 uptake as well as the NH_3 produced, it appears that the amino-sugars are first converted by oxidation into some product, probably an acid, which in turn undergoes deamination. It was found that glucosaminic acid cannot be regarded as the oxidation product responsible for the NH_3 accumulation. Glucosaminic acid was found to be stable in the presence of animal tissues, yeast and bacteria.

Attempts were made to identify the reaction products. So far, it has been established that there is no formation of lactic, acetic or succinic acid, that substances of ketonic or aldehyde character are absent, and that the products formed from amino-sugars are oxidized by muscle dehydrogenases in the presence of cozymase.

Iodoacetic acid, cinchophen and antiseptics completely abolish the activity of the amino-sugar enzyme. KCN and glyceraldehyde inhibit the oxidation, but have little effect on the NH_3 liberation. Glucose, unlike other sugars, considerably depresses deamination but interferes comparatively slightly with oxidation.

Part of the expenses of this research were defrayed by a grant from the Empire Rheumatism Council which the author wishes gratefully to acknowledge. Mr S. W. Williamson and Miss R. Leader rendered valuable assistance.

REFERENCES

Clift & Cook (1932). *Biochem. J.* **26**, 1788.
Cohen (1940). *J. biol. Chem.* **136**, 565.
Elson & Morgan (1933). *Biochem. J.* **27**, 1824.
György (1939). *J. biol. Chem.* **131**, 732.
Hagedorn & Jensen (1923). *Biochem. Z.* **135**, 46.
Levene & La Forge (1913). *J. biol. Chem.* **15**, 69.
Lutwak-Mann (1940, 1). *Biochem. J.* **34**, 517.
—— (1940, 2). *Chem. Ind.* **59**, 348.
Meyer & Smyth (1937). *J. biol. Chem.* **119**, 507.
Parnas & Heller (1924). *Biochem. Z.* **152**,
Pringsheim & Ruschmann (1915). *Ber. dtsch. chem. Ges.* **48**, 680.
White (1940). *J. chem. Soc.* p. 428.

65. THE METHYLATION OF WOOL WITH METHYL SULPHATE AND METHYL HALIDES

By STANLEY BLACKBURN, ERIC GEORGE HEMPHILL CARTER AND HENRY PHILLIPS

From the Wool Industries Research Association, Headingley, Leeds, 6

(*Received 19 April 1941*)

All the recognized methods of *O*- or *N*-methylation have been applied to proteins. Casein, for example, was boiled with CH_3I and KOH by Skraup & Krause [1909] and later treated with diazomethane by Geake & Nierenstein [1914]. Herzig & Landsteiner [1914] also used diazomethane to methylate casein, edestin and silk fibroin. The same authors [1918] later studied the methylation of wool with diazomethane and after eighteen treatments with this reagent obtained wool with a methoxyl content of 5·89 and 6·28 % of CH_3 linked to N. Treatment of wool with methyl alcoholic hydrochloric acid gave a methoxyl content of 3·80 and 1·49 % CH_3 as *N*-methyl. Edlbacher [1919] methylated a number of protamines with methyl sulphate and NaOH and determined the number of *N*-methyl but not of *O*-methyl groups. The conclusions drawn from these investigations were rendered doubtful by the discovery of methionine by Mueller [1921]. The *S*-methyl group of this acid yields CH_3I when boiled with HI and thus contributed to the CH_3 content of some of the methylated proteins prepared before 1921. More recently, Felix & Rauch [1931] and Felix & Reindl [1932] have prepared methyl esters of proteins by the action of dry HCl on their suspensions in CH_3OH. They found that the products could be further methylated by either methyl sulphate at *p*H 8 or 9, or by diazomethane. These more fully methylated proteins did not give the Pauly diazo reaction for the free iminazole ring or the Millon reaction for tyrosine. Haurowitz [1938] methylated egg albumin and horse haemoglobin with methyl sulphate. Rutherford *et al.* [1940] have recently made an extensive investigation of the methylation of silk fibroin with diazomethane.

Whilst attempting to methylate wool to obtain evidence of the existence of free thiol groups, we have found that it is possible to introduce CH_3 groups into wool keratin by using methyl sulphate, CH_3I or CH_3Br at ordinary temperatures. Evidence is given below that this reaction is largely, but not entirely, an esterification and appears to take place only when the wool is within its isoelectric region. Some evidence has been obtained that *N*-alkylation occurs at the same time.

Experimental

Unless otherwise stated, the wool used was in the form of a loosely woven flannel, similar to that described in previous investigations [Elsworth & Phillips, 1938].

Determination of CH_3 linked to O and S. All the methylated wools were analysed by Baernstein's method [1932; 1936] in which CH_3 groups linked to O and S, but not to N, are removed as CH_3I. Baernstein [1932] found that eleven different amino-acids and also glucose, when tested by this method, did not yield any volatile iodide. A similar result was obtained, during the present work, with methylamine. In addition, Barritt [1934] and Bailey [1937], using the method to determine the methionine in wool, were able to show that the sum of the cystine- and methionine-S was almost equal to the total S, suggesting that the method, when applied to wool, does not yield volatile iodide from unexpected sources.

Methylation of wool with methyl sulphate

(*a*) *Influence of* p*H on the reaction.* The wool used in these experiments was in the form of a worsted serge and had been scoured commercially. It was extracted with light petroleum and methylated by shaking for 90 min. with solutions of methyl sulphate in various buffers; 20 ml. of buffer containing 0·2 ml. of methyl sulphate per g. of conditioned[1] wool were used. At the end of each methylation the wool was rinsed in six changes of water, passed through a wringer after each rinse, dried and conditioned. The CH_3 content of the unmethylated wool was 0·06 %. The CH_3 contents of the methylated wools, together with the *p*H of the methylating solutions at the beginning and at the end of each methylation are given in Table 1. The experimental conditions chosen for these methylations were not ideal, since the *p*H of some of the solutions changed too much. The results suggest, however, that the maximum degree of methylation is attained when the *p*H of the methylating solution at the end of the treatment is within the range 2·5–8·5.

Table 1. *The influence of* p*H on the methylation of wool with methyl sulphate*

Buffer	Initial *p*H	Final *p*H	CH_3 %	Total CH_3, less methionine-CH_3 %
0·02*N* H_2SO_4	—	—	0·13	0·07
Phosphate	6·0	2·0	0·29	0·23
,,	7·0	2·5	0·37	0·31
,,	8·0	2·8	0·34	0·28
Borate	9·0	8·1	0·34	0·28
,,	10·0	8·6	0·35	0·29
,,	12·0	7·0	0·37	0·31
Acetate	12·0	9·0	0·13	0·07
,,	12·0	11·8	0·06	0·00

(*b*) *Repeated methylation with methyl sulphate.* The above experiments established optimum *p*H conditions for methylating wool, using a limited amount of methyl sulphate. Repetitions of these treatments were now made to see whether an upper limit existed to the amount of methylation which could be produced in this way. Wool was shaken for 90 min. with phosphate buffer, 0·15*M* with respect to H_3PO_4 (20 ml./g.) and methyl sulphate (0·2 ml./g.). The *p*H of the solution fell during this time from 7·6 to 6·3. A sample of the wool (3–4 g.) was then removed, rinsed with water, as previously described, dried, conditioned and analysed. The remaining wool was methylated again for the same length of time with fresh buffer and methyl sulphate. The CH_3 contents of wool methylated up to twenty times in this manner are plotted against the number of methylations in Fig. 1. This figure also contains a curve summarizing the results obtained when another sample of wool was treated with methyl sulphate (0·2 ml./g.) and sodium acetate (0·35 g./g. in 15 ml. water), these conditions being somewhat more drastic than when phosphate buffer was used. At 90 min. intervals small samples of wool were withdrawn for analysis and a further 0·2 ml. methyl sulphate and 0·35 g. sodium acetate added to the original solution. This was repeated nine times and then, after the wool had been washed and dried, for a further eight times. In the phosphate buffer, the initial rate of reaction was fairly rapid; in the acetate buffer, the rate of reaction was more uniform. In both buffers, however, the methylation appears to be reaching a limiting value of 1·9–2·0 % CH_3.

---

[1] All the wools described in this paper as conditioned had been exposed to a controlled atmosphere of 21° and 70 % R.H. until they attained a constant weight. It was found that methylation did not alter the moisture content (14 %), which was reached in this atmosphere at equilibrium. Although all the analyses were made on conditioned wool, the results are recorded as percentages of the anhydrous wool.

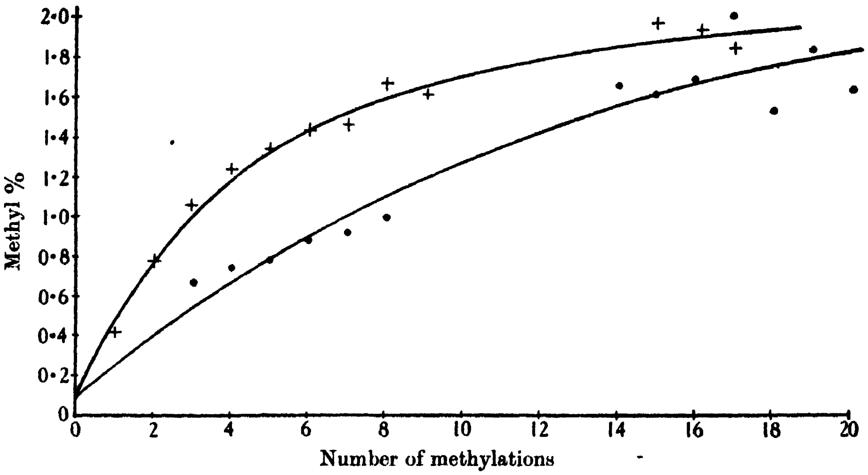


Fig. 1. The methylation of wool with methyl sulphate in phosphate and acetate buffers. +—+ Acetate buffer. •—• Phosphate buffer.

Methylation of wool with methyl iodide and methyl bromide

(*a*) *Methylation of wool with CH_3I.* For reasons that will become apparent later, it was thought that if wool reacted at ordinary temperatures with methyl sulphate it should also react with methyl iodide. Conditioned wool (1 part) was placed in a bottle containing CH_3I (6 parts), and kept at ordinary temperatures in the dark. At intervals samples of the wool were withdrawn, washed with benzene and then extracted with benzene in a Soxhlet apparatus, quinoline being placed in the distillation flask to retain the extracted CH_3I. The CH_3 content of the extracted wool was determined. A portion of the benzene-extracted wool was boiled with water through which steam was passed for 1 hr., dried, conditioned, and the CH_3 content redetermined. Another portion of the benzene-extracted wool (1·5 g.) was shaken with phosphate buffer (*p*H 6·0, 75 ml.) for 3½ hr., rinsed in water, dried, conditioned and analysed. In Table 2, the CH_3 contents of wools that had been methylated with CH_3I for increasing periods of time and then extracted with benzene are recorded, together with the CH_3 contents of the extracted wools after distillation in steam and also after extraction with phosphate buffer. From these results it is apparent that after the methylated wool has been extracted with benzene, subsequent distillation in steam or extraction with phosphate buffer causes only small decreases in its CH_3 content.

Table 2. *Percentage CH_3 content of wool attained at ordinary temperatures with CH_3I*

Time of exposure to CH_3I, hr. ...	2	6	17	24	90	144	336
(i) After extraction with benzene	0·38	0·30	0·38	0·43	0·53	0·41	0·45
(ii) After extraction with benzene followed by steam distillation for 1 hr.	0·36	0·24	—	0·36	0·49	0·35	0·44
(iii) After extraction with benzene followed by extraction with buffer *p*H 6·0	0·36	0·23	0·36	0·39	0·52	0·40	—

It appears that wool reacts fairly rapidly with CH_3I, and that prolonged periods of exposure cause only a slight further reaction. The irregular variation in the analyses after different times of exposure is most likely due to slight variation in the samples of wool taken for each experiment, and the CH_3 figures after, say, 2 days may be taken to be sensibly constant. Another irregularity that has been observed is that the rate of reaction may vary within wide limits. For example, another sample of wool brought to equilibrium with a phosphate buffer of *p*H 5·9, and then dried and conditioned, at first reacted much more slowly; after 2 hr. exposure to CH_3I its CH_3 content was 0·09 %, after 24 hr. 0·19 %, but after 144 hr. 0·45 %. The reaction appears to depend on the physico-chemical condition of the wool. Thus when conditioned wool was heated under reflux with CH_3I for 2 hr., it contained 0·30 % CH_3 after benzene extraction. On the other hand, when the CH_3I was diluted with an equal volume of ether, and the wool heated under reflux for 2 hr., its CH_3 content, after benzene extraction, was 0·14 %. Dried wool reacted with CH_3I very slowly; after 72 hr. exposure, the wool contained only 0·09 %, i.e. only slightly more than that due to methionine (0·06 %).

Presumably when wool is methylated with CH_3I, for each CH_3 group introduced, one atom of anionic iodine is liberated. The determination of this anionic iodine thus provides an additional measure of the degree of methylation that occurs. The samples (1·5 g.) of methylated and benzene-extracted wools, the analyses of which are given in Table 2, were shaken for 3½ hr. with 75 ml. of buffer, *p*H 6·0. These conditions were chosen as the result of separate experiments which showed that this method gave the maximum extraction of iodide. The filtered buffer and washings from the wool were acidified with dilute HNO_3, the iodide precipitated with silver nitrate and the excess silver nitrate determined with potassium thiocyanate. The results are recorded in Table 3, in which CH_3 equivalent to the iodide is also given. By subtracting the methionine-CH_3 (0·06 %) from the figures given for the total CH_3 in Table 2, the figure for the total *O*-methyl is obtained. This is seen to be less than the CH_3 equivalent of the anionic iodide found. The extra methylation that occurs, as revealed by the

Table 3. *Anionic iodine contents of wool exposed to CH_3I for increasing periods of time and then extracted with benzene*

Time of exposure hr.	Anionic iodine %	CH_3, equivalent to anionic I found %	Observed CH_3, less methionine-CH_3	Excess CH_3 (calc.) %
2	3·68	0·43	0·32	0·11
6	3·46	0·41	0·24	0·17
17	3·83	0·45	0·32	0·13
24	3·98	0·47	0·37	0·10
90	5·30	0·63	0·47	0·16
144	3·83	0·45	0·35	0·10

iodide figures, probably represents N-methylation which is not included in the CH_3 as determined by the Baernstein method. This excess CH_3 is given in the fifth column of Table 3.

(*b*) *Repeated methylation of wool with CH_3I.* The wool (30 g.) was exposed to CH_3I, as in the previous experiments, except that the operation was repeated four times at intervals, recorded in Table 4. Before each exposure, the wool was buffered to *p*H 7·9 with borate buffer.

Table 4. *The CH_3 contents of wool after repeated exposures to CH_3I*

Time of exposure days	CH_3 %	I %
14	0·52	4·77
28	0·80	—
46	1·02	—
88	1·06	—

After hydrolysis for 24 hr. with 5N HCl and evaporation *in vacuo*, the CH_3 content of the hydrolysate was 0·10 %.

(*c*) *Methylation of wool with CH_3Br.* Conditioned wool (1 part), buffered to *p*H 7·7 in borate, was treated with CH_3Br (1·5 parts) in a tightly stoppered bottle at room temperature. At intervals, the pressure was released, the CH_3Br allowed to evaporate and the wool conditioned and analysed. The results obtained are given in Table 5.

When wool, methylated in this manner and having 1·30 % CH_3, was subjected to complete hydrolysis with HCl, the CH_3 content of the hydrolysate was 0·08 %. It is noteworthy that the 'excess' CH_3 introduced by the action of CH_3Br is greater than that obtained using CH_3I.

Table 5. *Methylation of wool with CH_3Br*

Total time of exposure, days	CH_3 %	Br %	'Excess' CH_3 (calc.), %
1	0·29	—	—
4	0·58	4·98	0·42
(CH_3Br renewed)			
6	0·65	—	—
18	0·92	7·53	0·55
(CH_3Br renewed)			
44	1·30	—	—
(Re-buffered and CH_3Br renewed)			

(*d*) *Methylation of silk.* Because of the difference in chemical constitution between wool and silk, it was of interest to study the methylation of silk in a

Table 6. *Methylation of silk*

(*a*) With methyl sulphate (phosphate buffer).

No. of methylations	CH_3, %
2	0·20
5	0·40
9	0·45
11	0·46
13	0·50
14	0·45

(*b*) With CH_3I

Time of exposure days	CH_3 %	I %	CH_3, equivalent to I found %	'Excess' CH_3 (calc.) %
19	0·37	4·72	0·56	0·19

similar manner. Silk, in the form of an unweighted fabric, was methylated with methyl sulphate in a phosphate buffer, and with CH_3I, using the conditions previously described for wool. The results are given in Table 6.

Stability of the methoxyl groups introduced

The CH_3 content found by analysis after these treatments might conceivably have been due to retention of the methylating reagent in the wool without any definite chemical reaction having occurred. Experiments were made to determine whether the CH_3 content could be reduced by certain mild treatments.

(i) After heating in a strong stream of air at 115° for 2 hr., samples of wool methylated with methyl sulphate and containing 0·30 and 1·62 % CH_3 were found to give unchanged analyses, although the same treatment for 10 min. would remove 14 % of water from conditioned wool. Further, wool methylated with CH_3I and then extracted with benzene contained 0·30 % CH_3 after heating in a current of air at 115° for 30 min. and 0·29 % after 60 min.

(ii) In order to obtain evidence of the degree of resistance to hydrolysis of the methyl groups which had been introduced, the wool, methylated with methyl sulphate, was steam distilled. Wool (2 g.) containing 0·36 % CH_3 was covered with water (20 ml. *p*H 8) and steam was blown into the water for the times given in Table 7. At the end of each distillation, a sample of the wool was dried, conditioned and then analysed. The analyses show that the CH_3 is held tenaciously by the wool, although it is gradually lost as the time of distillation increases. The results of a similar series of experiments on methylated wools of higher CH_3 contents are also given in Table 7. In these experiments, before the steam was blown into the flask, the methylated wool was covered with 5 ml. of a borate buffer (*p*H 7) and 15 ml. water, in order to prevent a large alteration in *p*H during the distillation. A rapid drop in the CH_3 content of these wools is observed at the beginning of the distillation, followed by a smaller drop as distillation is continued. For a 'fully methylated' wool, the CH_3 groups introduced above a certain value (about 0·6–0·7 % CH_3) are much more readily removed by distillation than those below this value. This may indicate that there are two centres which are being methylated, from one of which the CH_3 groups are readily removed, whilst the CH_3 groups attached to the other resist hydrolysis under the conditions employed. This point is discussed more fully later.

Table 7. *The effect of exposing wool, methylated with methyl sulphate, to a current of steam*

	Duration of steam distillation, min.								
	0	5	10	20	40	60	120	180	240
CH_3, %	—	0·28	0·28	0·24	0·23	0·27	0·22	—	0·16
	0·31	—	—	—	—	0·23	0·20	0·19	0·16
	0·66	—	—	—	—	0·49	0·39	0·38	0·31
	1·01	—	—	—	—	0·64	0·59	0·58	0·46
	1·07	—	—	—	—	0·75	0·67	0·65	0·58
	1·63	—	—	—	—	0·72	0·71	0·57	0·55

(iii) When wool (CH_3, 0·37 %) that had been methylated once with methyl sulphate and buffer was placed in a 0·5 % solution of sodium sesquicarbonate (*p*H 10·0) or in 0·1 *N* NaOH, and steam was blown through the water, the CH_3 was quickly removed and in 30 min. the CH_3 content of the wool returned to its pre-methylation value of 0·06 %. This experiment provided clear evidence that the CH_3 was linked to the wool by a readily hydrolysable linkage and was not, therefore, attached either to the phenolic group of tyrosine or to the S of cysteine.

Similar results were obtained with more highly methylated wools. A wool containing 1·62 % CH_3 gave a value of 0·21 % CH_3 after distillation with 1 % sodium sesquicarbonate (*p*H 10) for 1 hr. When the wool was distilled in a current of steam for 1 hr. and then treated with sodium sesquicarbonate, the final CH_3 content was 0·10 %. It is evident that even after repeated treatment with methyl sulphate the number of methoxyl groups which are attached to the wool by non-hydrolysable linkages is extremely small. When the wool was subjected to complete hydrolysis (24 hr.) with HCl, the CH_3 content of the hydrolysate, determined after evaporation *in vacuo*, corresponded to the methionine content of the original wool.

(iv) Experiments were made to determine the ease with which the CH_3 of methylated wool could be removed by extraction with solutions of different *p*H. Samples of wool (2·0 g., CH_3, 1·66 %) methylated with methyl sulphate and buffer were treated with 100 ml. of 'Universal' buffer of different *p*H (containing acetic, phosphoric and boric acids and NaOH) at room temperature in closed bottles. After standing overnight, the wools were washed once with water, dried, conditioned and analysed. The *p*H of the solutions from which the wools had been taken was determined with a glass electrode. The results are recorded in Table 8.

Table 8. *The effect of extracting methylated wool with buffers of different* pH

Initial *p*H	0·46	1·8	2·82	4·59	6·27	8·38	9·46	10·45
Final *p*H	0·75	1·86	2·99	4·64	6·32	8·10	9·03	9·44
Residual CH_3, %	1·09	1·44	1·40	1·32	1·24	1·15	0·82	0·87

Titration curve of wool after methylation

(*a*) *Wool methylated with methyl sulphate.* A large sample of wool fabric was methylated in buffer at *p*H 6 and on analysis contained 0·32 % CH_3 and 0·21 % after steam distillation for 60 min. The wool was remethylated and then contained 0·30 % CH_3 after distillation in steam. The disulphide contents of the wool before and after methylation were 2·93 and 2·88 %, respectively. Samples of the methylated and unmethylated wool fabrics were washed with distilled water and finally brought to equilibrium with water at *p*H 5. Each fabric was cut up into about 150 pieces, and samples (1–2 g.) of each weighed and put into bottles containing either solutions of H_2SO_4 or NaOH in which they were kept with occasional shaking for 6 days. The *p*H of the initial and final solutions was determined with the glass electrode. The more concentrated solutions were titrated, using bromocresol purple as indicator. Some of the more alkaline solutions developed an odour resembling that of the methylamines. The results obtained are plotted as ml. *N* acid or alkali absorbed by 100 g. of anhydrous wool in Fig. 2.

In order to detect any possible alteration in the water-holding capacity of the wool caused by methylation, which would have affected the calculations used to construct the curves of Fig. 2, samples of the wools were squeezed, dried at 65°, and then conditioned. The moisture content of each sample was determined by the method of Barritt & King [1926] by drying in a slow current of air at 110°. No appreciable difference was found between the moisture contents of the methylated and unmethylated wools.

(*b*) *Wool methylated with methyl iodide.* A further sample of wool, after buffering to *p*H 8 in borate buffer, was treated with CH_3I. After 11 days the wool was extracted with benzene and found to contain 0·59 % CH_3. The methylated and unmethylated wools were washed with distilled water, and brought into equilibrium with water at *p*H 5, the subsequent procedure being as described in (*a*) above. The results are plotted in Fig. 2.

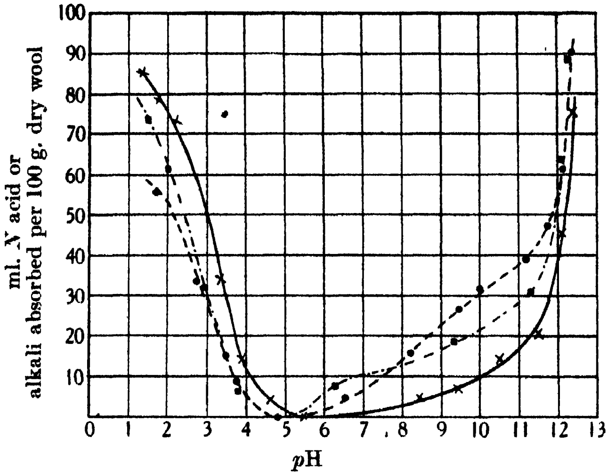


Fig. 2. The titration curves of wool before and after methylation with methyl sulphate and with methyl iodide. × — × Untreated. • – – • Methylated with CH_3I (0·59 % CH_3). ■—·—■ Methylated with methyl sulphate (0·25 % CH_3).

In agreement with the hypothesis that the wool is esterified by both methylating agents, less acid is required to suppress the ionization of the carboxyl groups of the wool after methylation, being equivalent, at *p*H 1·5, to 11·3 ml. *N* acid (i.e. 0·17 % CH_3) in the wool methylated with methyl sulphate, and to 23 ml. *N* acid (i.e. 0·32 % CH_3) in the wool methylated with methyl iodide. No quantitative conclusions can, however, be drawn from these curves since some of the CH_3 may have been lost when the methylated wools were purified. On the alkaline side of *p*H 5, it is important to notice that the two methylated wools behave differently. Although both wools neutralize an appreciable amount of alkali from *p*H 5 to 7, the wool methylated with methyl sulphate neutralizes more than the wool methylated with CH_3I. This alkali is neutralized during the back-titration of some feebly basic group. In the more alkaline solutions, the wool methylated with CH_3I neutralizes more alkali than the wool methylated with methyl sulphate. It is probable that this alkali is neutralized during the hydrolysis of carbomethoxyl groups. On this basis, whereas methylation with CH_3I favours esterification of carboxyl groups, methylation with methyl sulphate favours the production of the feebly basic groups.

DISCUSSION

There can be little doubt that the CH_3 groups found in the methylated wools described are chemically combined, since they persist after the wool has been extracted with benzene and water, subjected to steam distillation and exposed to a current of hot air. The clearest indication of their state of combination is given by the ease with which they can be removed from the wool by acid hydrolysis and by boiling it with buffer solutions higher than *p*H 9. In addition, when methylated wools are steam distilled in the presence of alkali, methyl alcohol can be detected in the distillate by Schryver & Wood's method [1920]. The removal of all the CH_3, with the exception of the methionine-CH_3, by this method indicates clearly that the added CH_3 is not derived from methylated thiol groups or from methylated tyrosine. Further, since the method used in the determinations of CH_3 is unlikely to return CH_3 attached to nitrogen, the CH_3 is not derived from methylimide groups. Some *N*-methylation occurs, however, when wool is methylated with CH_3I and CH_3Br, since the amount of anionic halide introduced is more than equivalent to the CH_3 as estimated by the Baernstein method.

When *O*-methylation takes place, the most probable reaction is the esterification of carboxyl groups. This conclusion is of particular interest since carboxylic acids are not esterified readily under the conditions used in the present experiments, by either methyl sulphate or methyl iodide. On the other hand, both these substances esterify the zwitterions of amino-acids [Taylor & Baker, 1937].

$$NH_3^+CH_2COO^- \xrightarrow{MeI} NH_3^+CH_2COOMe\ [I^-].$$

Engeland [1910], for example, found that phenylalanine was methylated on boiling with methyl alcoholic KOH and CH_3I, and Novak [1912] employed methyl sulphate and NaOH with a variety of amino-acids.

Free amino-acids do not, however, exist in wool in any appreciable quantities and therefore the methyl sulphate, iodide and bromide must esterify combined amino-acids. If these acids are monoaminomonocarboxy-acids, they may terminate the main polypeptide chains to which they must be linked through their amino groups. More probably they are monoaminodicarboxy-acids which are built into the polypeptide chain and esterify because their carboxyl groups terminating the side-chains form zwitterions with the amino groups terminating the side-chains derived from the monocarboxydiamino-acids. In other words, esterification occurs at salt linkages. Evidence for the existence of linkages of this nature in keratin has been accumulated by Speakman & Hirst [1931; 1933].

There are several indications that wool keratin will only esterify when within its isoelectric region. For example, the data given in Table 1 show that the highest degree of methylation was obtained when wool was methylated with solutions of methyl sulphate of *p*H 2·5–8·5. Further, anhydrous wool did not methylate during 75 hr. exposure to CH_3I, nor would conditioned wool methylate when boiled with an ethereal solution of CH_3I, although it underwent limited methylation when boiled with CH_3I. Moreover, when wool with a high CH_3 content is extracted with solutions of different *p*H, the CH_3 groups show the greatest stability in the range *p*H 2–8, solutions with *p*H values outside this range causing a much larger fall in the CH_3 content (Table 8).

The hypothesis that methylation of carboxyl groups occurs owing to the influence of the adjacent basic groups of histidine, lysine and arginine seems to be qualitatively adequate to explain the results already obtained. Amino-acid analyses of wool keratin are available from which it is possible to see the extent of the quantitative agreement between the CH_3 content of methylated wool and the CH_3 content that might be expected. In Table 9 the percentages of the amino-acids in wool likely, on the above hypothesis, to be involved in zwitterion formation are given, together with the equivalent amount of CH_3 to which they might give rise assuming that they form salt linkages.

The CH_3 content of wool should reach a maximum value equal to the free carboxyl groups, plus the CH_3 due to the *S*-methyl of the methionine present and to its probable conversion into a sulphonium group. The free carboxyl

Table 9. *The composition of wool*

Amino-acid		% of wool	Equivalent CH_3, % of wool
Arginine	Vickery [1940]	10·4	0·90
	Marston [1928]	10·2	0·88
Lysine	Vickery & Block [1930]	2·3	0·24
	Block [1939]	2·5	0·26
	Marston [1928]	2·8	0·29
Histidine	Vickery & Block [1930]	0·7	0·07
Glutamic acid	Abderhalden & Voitinovici [1907]	12·9	1·32
	Speakman & Townend [1937]	15·3	1·56
Aspartic acid	Abderhalden & Voitinovici [1907]	2·3	0·26
	Speakman & Townend [1937]	7·3	0·82
Methionine	This paper	0·6	0·12*
Amide-N	Speakman & Townend [1937]	1·37	1·47

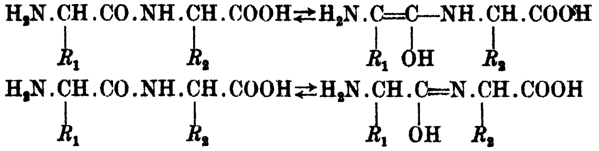
* Assuming methionine combines with the methylating agent to give a sulphonium compound.

groups of wool are limited to the carboxyl groups of glutamic and aspartic acids which are not linked in the main chain or combined with ammonia as amide groups. Using the figures of Abderhalden & Voitinovici [1907] for glutamic and aspartic acids and Speakman & Townend's [1937] figure for amide-N, the CH_3 content of fully methylated wool might have a possible value of $1{\cdot}58-1{\cdot}47+0{\cdot}06=0{\cdot}17\%$. If the later figures of Speakman & Townend for glutamic and aspartic acids are utilized, the CH_3 content would be 0·96%. Assuming that CH_3I (or sulphate) adds on to the sulphur atom of methionine [Toennies, 1940, 1, 2], these values would be increased by 0·06%. The number of CH_3 groups that wool can take up is much larger than even the higher of these figures.

It would seem to be improbable that appreciable hydrolysis of the peptide links of the main chains, liberating further carboxyl groups which might then undergo methylation, would occur during even prolonged treatment at the *p*H employed in these experiments. Moreover, hydrolysis of this type could hardly occur during treatment of wool with CH_3I or CH_3Br, and yet one wool methylated with CH_3Br contained more CH_3 (1·30%) than could be accommodated by the free carboxyl groups.

The results obtained on methylating silk fibroin are of importance in this connexion. It can be seen from Table 6 that when the methods used for wool are applied to silk a maximum value of 0·45% of CH_3 is reached. The greater part of this CH_3 is removed on treatment with alkali. Rutherford *et al.* [1940] have recently obtained similar results after methylating silk with diazomethane. These authors found that the free phenolic groups of tyrosine were readily methylated, but in addition the total methoxyl content was found to include some 'extra' methoxyl groups, corresponding to 0·26 millimols/g. (or 0·39% CH_3) which were removed as CH_3OH on alkaline hydrolysis and were not attached to primary amino or serine hydroxyl groups. These authors tentatively assumed that the 'extra' groups were located on carboxyl groups. Since, as far as we are aware, no one has succeeded in isolating dicarboxylic amino-acids from silk fibroin, the amounts of dicarboxylic acids present in silk are likely to be extremely small. The diamino-acid content of silk is also very low: Vickery & Block [1931] found 0·74% arginine, 0·25% lysine and 0·076% histidine. Salt linkage formation between the basic side-chains of these acids and free carboxyl groups derived from other acids could therefore only give rise to zwitterions capable of being combined with 0·10% CH_3.

The esterification of carboxyl groups cannot account for all the hydrolysable CH_3 with which wools and silk will combine. In methylated wool as much as 0·7 %, and in methylated silk as much as 0·4 % CH_3, are probably linked to oxygen atoms which are not present in carboxyl groups. We are therefore forced to entertain the possibility that the oxygen atoms to which this CH_3 is attached are the oxygen atoms of the peptide linkages. Several workers have discussed the possibility of an enolization in peptides and proteins, which can theoretically occur in two ways:



$$H_2N.\underset{R_1}{\underset{|}{CH}}.CO.NH.\underset{R_2}{\underset{|}{CH}}.COOH \rightleftarrows H_2N.\underset{R_1}{\underset{|}{C}}{=}\underset{OH}{\underset{|}{C}}{-}NH.\underset{R_2}{\underset{|}{CH}}.COOH$$

$$H_2N.\underset{R_1}{\underset{|}{CH}}.CO.NH.\underset{R_2}{\underset{|}{CH}}.COOH \rightleftarrows H_2N.\underset{R_1}{\underset{|}{CH}}.\underset{OH}{\underset{|}{C}}{=}N.\underset{R_2}{\underset{|}{CH}}.COOH$$

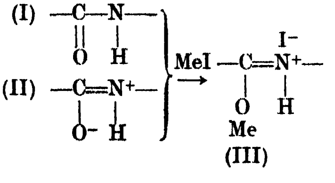
Enolization of the first type involving loss of asymmetry in the carbon atom was postulated by Dakin [1912–13] to explain the racemization of certain amino-acids in proteins by the action of alkali at low temperatures. The idea was later elaborated by Levene and co-workers [Levene *et al.* 1927; Levene *et al.* 1931; Levene & Yang, 1933] in a study of the racemization and hydrolysis of peptides under the influence of alkali. Levene also expressed the opinion that enolization of the second type, involving the nitrogen atom, could occur in polypeptides and proteins.

Moreover, it is possible to obtain stable isomers of compounds containing the peptide linkage. This isomerism has been shown to be due to enolization. Thus Leuchs & Manasse [1907] demonstrated the existence of two isomeric forms of glycylglycine carboxylic acid, the 'α' form having the lactam formula, the 'β' having the lactim formula:

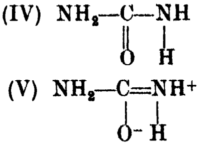
$$HOOC.NH.CH_2.CONH.CH_2.COOH \qquad \alpha$$
$$HOOC.NH.CH_2.C(OH){=}N.CH_2.COOH \qquad \beta$$

In accordance with expectation, when the enolizable hydrogen atom is replaced by a phenyl group, only the 'α' form is obtained. Leuchs & La Forge [1908] have prepared similar isomers of *N*-carboxydiglycylglycine.

If one or more of the peptide links in wool or silk can readily enolize, methylation might be expected to occur. It is not, however, necessary to assume that the imido-hydrogen atom migrates. More probably the methylating agent adds on to a peptide linkage which has become a resonance hybrid of the two structures (I) and (II) and as such, since it approaches a zwitterion in constitution, might conceivably methylate in a similar manner.



$$\left.\begin{matrix}(I)\ -\underset{O}{\underset{\|}{C}}-\underset{H}{\underset{|}{N}}- \\ (II)\ -\underset{O^-}{\underset{|}{C}}{=}\underset{H}{\underset{|}{N^+}}-\end{matrix}\right\} \overset{MeI}{\longrightarrow} -\underset{\underset{Me}{O}}{\underset{|}{C}}{=}\overset{I^-}{\underset{H}{\underset{|}{N^+}}}- \quad (III)$$

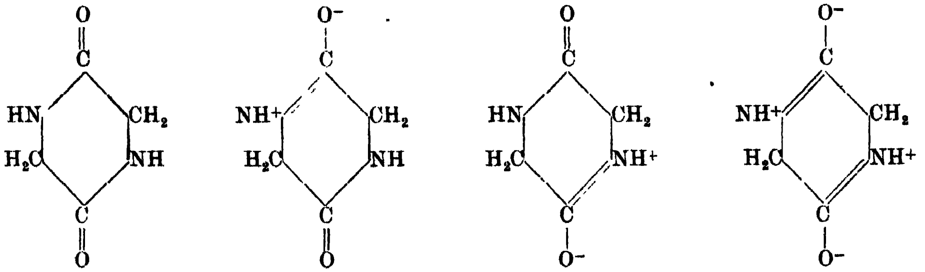


$$(IV)\ NH_2-\underset{O}{\underset{\|}{C}}-\underset{H}{\underset{|}{NH}}$$
$$(V)\ NH_2-\underset{O^-}{\underset{|}{C}}{=}\underset{H}{\underset{|}{NH^+}}$$

In support of this view, urea which undergoes methylation on the oxygen atom when treated with methyl sulphate has been considered to be a resonance-hybrid of the two forms (IV) and (V) [Taylor & Baker, 1937].

On the chemical side, two pieces of evidence are in agreement with the presence of methylated peptide linkages possibly possessing the structure indicated in (III). The first is the lability of some of the CH_3 in the highly methylated wools, and secondly, the neutralization of alkali by methylated wools from *p*H 5 to 7 (Fig. 2). An imido-group, which had undergone salt formation as indicated in (III), would be expected to hydrolyse freely and to lose HI within this *p*H range.

Physical evidence supporting the possible occurrence of 'activated' peptide linkages is also available. From the results of an X-ray examination of the crystal structure of diketopiperazine, Corey [1938] concluded that the diketopiperazine molecule was a resonance hybrid of four possible structures, three of which contain one or more zwitterionic peptide linkages.



Ellis & Bath [1939] found that the infra-red absorption spectrum of diketopiperazine also supported Corey's idea of electron resonance. Karrer *et al.* [1923] obtained a derivative of a tautomeric form of diketopiperazine in which the hydrogen atom is enolized in the above sense. These authors found that when the silver derivative of diketopiperazine reacted with benzyl chloride, a benzyl group was attached to the oxygen atom of the peptide bond, 2:5-dihydroxydihydropyrazine dibenzyl ether being produced. This compound was readily hydrolysed by dilute acids to benzyl alcohol and glycine. Methylation would not be expected to occur at all the peptide linkages in wool and silk, but only at certain specific links which can assume the appropriate electronic state, or, some state approaching it electronically, due to the presence of adjacent activating groups.

The total CH_3 content of methylated wool may thus include two types of *O*-methyl groups: one attached to the free carboxyl groups of glutamic and aspartic acids, the other attached to peptide linkages. On this hypothesis, a large proportion of the CH_3 groups in methylated silk would be attached to peptide linkages. Further work is in progress to enable a clearer distinction to be drawn between methylation at these two centres.

SUMMARY

1. Wool can be methylated with methyl sulphate, iodide and bromide, at ordinary temperatures.

2. With each reagent, both *O*- and *N*-methylation takes place.

3. The experimental results indicate that the *O*-methylation consists partly of an esterification of the free carboxyl groups.

4. Evidence is presented which suggests that *O*-methylation also takes place at peptide linkages.

Thanks are due to the Council of the Wool Industries Research Association for permission to publish this paper, to Dr L. F. Story for determining the titration curves given in Fig. 2, and to Mr G. Lindley and Mr A. B. Gibson for assistance with the experimental work.

REFERENCES

Abderhalden & Voitinovici (1907). *Hoppe-Seyl. Z.* **52**, 348.
Baernstein (1932). *J. biol. Chem.* **97**, 663.
—— (1936). *J. biol. Chem.* **115**, 25.
Bailey (1937). *Biochem. J.* **31**, 1396.
Barritt (1934). *Biochem. J.* **28**, 1.
—— & King (1926). *J. Text. Inst. Manchr*, **17**, 386.
Block (1939). *J. biol. Chem.* **128**, 181.
Corey (1938). *J. Amer. chem. Soc.* **60**, 1598.
Dakin (1912–13). *J. biol. Chem.* **13**, 362.
Edlbacher (1919). *Hoppe-Seyl. Z.* **107**, 52.
Ellis & Bath (1939). *J. chem. phys.* **7**, 862.
Elsworth & Phillips (1938). *Biochem. J.* **32**, 837.
Engeland (1910). *Ber. dtsch. chem. Ges.* **43**, 2662.
Felix & Rauch (1931). *Hoppe-Seyl. Z.* **200**, 27.
—— & Reindl (1932). *Hoppe-Seyl. Z.* **205**, 11.
Geake & Nierenstein (1914). *Biochem. J.* **8**, 287.
Haurowitz (1938). *Hoppe-Seyl. Z.* **256**, 28.
Herzig & Landsteiner (1914). *Biochem. Z.* **61**, 458.
—— —— (1918). *Mh. chem.* **39**, 369.
Karrer, Granacher & Schlosser (1923). *Helv. chim. Acta*, **6**, 1108.
Leuchs & La Forge (1908). *Ber. dtsch. chem. Ges.* **41**, 2586.
—— & Manasse (1907). *Ber. dtsch. chem. Ges.* **40**, 3235.
Levene, Bass, Steiger & Bencowitz (1927). *J. biol. Chem.* **72**, 815.
—— Steiger & Marker (1931). *J. biol. Chem.* **93**, 605.
—— & Yang (1933). *J. biol. Chem.* **99**, 405.
Marston (1928). *Bull. Coun. sci. industr. Res., Aust.*, **38**.
Mueller (1921). *Proc. Soc. exp. Biol., N.Y.*, **18**, 14.
Novak (1912). *Ber. dtsch. chem. Ges.* **45**, 834.
Rutherford, Patterson & Harris (1940). *Amer. Dyest. Rep.* **29**, 583.
Schryver & Wood (1920). *Analyst*, **45**, 164.
Skraup & Krause (1909). *Mh. Chem.* **30**, 447.
Speakman & Hirst (1931). *Nature, Lond.*, **128**, 1073.
—— —— (1933). *Trans. Faraday Soc.* **29**, 148.
—— & Townend (1937). *Nature, Lond.*, **139**, 411.
Taylor & Baker (1937). Sidgwick's Organic Chemistry of Nitrogen, p. 284. Oxford.
Toennies (1940, 1). *Proc. Amer. Soc. biol. Chem.* **34**, cii (*J. biol. Chem.* **133**).
—— (1940, 2). *J. biol. Chem.* **132**, 455.
Vickery (1940). *J. biol. Chem.* **132**, 325.
—— & Block (1930). *J. biol. Chem.* **86**, 107.
—— —— (1931). *J. biol. Chem.* **93**, 105.

66. THE ISOLATION OF AMINO-ACIDS FROM RUBBER LATEX

By G. S. WHITBY and H. GREENBERG

From the Department of Chemistry, McGill University, Montreal

(*Received 21 May 1941*)

After an examination of the chemical composition of the 'resin' of rubber had been carried through [Whitby *et al.* 1926] there was initiated a chemical examination of the aqueous serum left on separating rubber from the latex of *Hevea brasiliensis*. This latter was carried as far as a study of the amino-acids present, and had then to be interrupted. As it has not been possible since to resume it, and as several papers on the amino-acid composition of the proteins of *Hevea* latex have recently appeared, it is now thought desirable to put on record the results which were obtained.

While several investigations, mentioned below, have been made of the amino-acids derived by hydrolysis from the proteins of rubber and latex serum, only one previously published investigation has been concerned with the amino-acids present as such in latex. McGavack & Rumbold [1934] isolated *d*-alanine from latex serum and secured some evidence of the presence of phenylalanine. In the present investigation the following seven amino-acids were isolated from the serum: tyrosine, *l*-leucine, *d*-*iso*leucine, *d*-valine, *d*-arginine, *l*-aspartic acid and *i*-proline. Indications were also obtained of the presence of phenylalanine.

It may be noted that one of these, *d*-valine, was previously isolated from the acetone extract of rubber (sheet form) [Whitby *et al.* 1926]. Clearly, it had been taken up by the coagulum from the serum and had escaped complete removal in the preparation of the rubber.

It is interesting to compare these findings as to the free amino-acids present in *Hevea* latex with the results of the examination by various investigators of the amino-acids obtained by the hydrolysis of protein from rubber and serum. Belgrave [1925, 1] obtained evidence of the presence of glycine and proline in proteins obtained (*a*) from latex serum by precipitation with alcohol, (*b*) from rubber by exhaustive extraction with benzene. Later work by Belgrave [1925, 2] on the former protein indicated the presence, in addition, of arginine (in high proportion), cystine, histidine and lysine. Midgley *et al.* [1937] obtained the following from protein isolated from rubber: glycine, proline, arginine, histidine, lysine, leucine, aspartic acid and a representative of the group comprising alanine, phenylalanine, hydroxproline and serine. They state that the following were definitely absent: cystine, glutamic acid and tyrosine. Subsequent authors, however, working it is true with protein isolated from rubber or latex in different ways, have identified these three amino-acids in hydrolysates.

In protein obtained from latex serum by precipitation with ammonium sulphate, Altman [1935] identified and estimated proline, arginine, histidine, leucine, aspartic acid, alanine, dihydroxyphenylalanine, tyrosine, glutamic acid, hydroxyproline and valine. Tristram [1940] analysed protein obtained by extraction of dried, fresh latex for the following nine amino-acids, all of which were present and which in total constituted about 50 % of the protein: arginine, histidine, leucine, aspartic acid, tyrosine, glutamic acid, cystine, methionine and tryptophan.

Table 1. *Amino-acids in latex and from latex proteins*

Proteins isolated by
1. Precipitation of serum with alcohol.
2. Precipitation of serum with ammonium sulphate.
3. Heat coagulation of serum.
4. Extraction of dried latex.
5. Separation from rubber.
6. Separation from rubber.

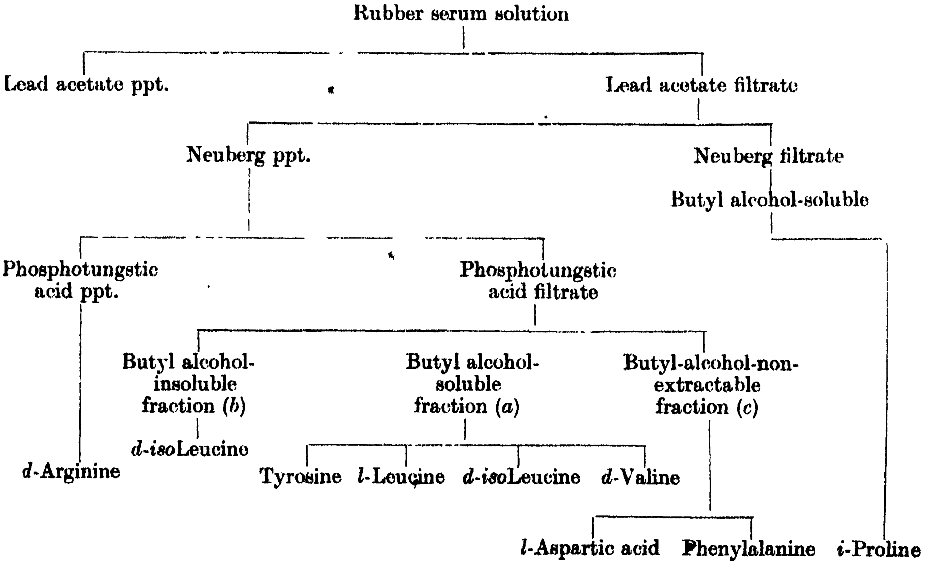
In latex serum		By hydrolysis of proteins					
McGavack & Rumbold [1934]	Present authors	1 Belgrave [1925, 1, 2]	2 Altman [1935]	3 Present authors	4 Tristram [1940]	5 Belgrave [1925, 1]	6 Midgley *et al.* [1937]
—	—	Glycine	—	Glycine	—	Glycine	Glycine
Alanine	—	—	Alanine	—	—	—	—
—	Valine	—	Valine	—	—	—	—
—	Leucine	—	Leucine	—	Leucine	—	Leucine
—	*iso*Leucine	—	—	—	—	—	—
Phenylalanine	Phenylalanine	—	Dihydroxyphenylalanine	—	—	—	—
—	Tyrosine	—	Tyrosine	Tyrosine	Tyrosine	—	—
—	Aspartic acid	—	Aspartic acid	Aspartic acid	Aspartic acid	—	Aspartic acid
—	—	—	Glutamic acid	Glutamic acid	Glutamic acid	—	—
—	Arginine	Arginine	Arginine	Arginine	Arginine	—	Arginine
—	—	Histidine	Histidine	Histidine	Histidine	—	Histidine
—	—	Lysine	—	Lysine	—	—	Lysine
—	Proline	Proline	Proline	Proline	—	Proline	Proline
—	—	—	Hydroxyproline	—	Tryptophan	—	—
—	—	Cystine	—	—	Cystine	—	—
—	—	—	—	—	Methionine	—	—

In the present work a cursory examination of the heat-coagulable protein of latex serum led to the isolation from its hydrolysate of glycine, proline, aspartic acid, glutamic acid and tyrosine, and gave evidence of the presence, in addition, of histidine, arginine and lysine.

Table 1 summarizes the results of investigations made to date on the identification, isolation and estimation of the amino-acids of rubber, latex and latex serum.

In isolating, in the present investigation, amino-acids from latex serum, the method of fractionation employed was essentially that of Vickery [1924; 1925] and of Vickery & Leavenworth [1925], in which use is made of the mercuric acetate-sodium carbonate reagent of Neuberg & Kerb [1912] to precipitate the amino-acids as their carbonates and the purines as mercury complexes. The chief modification introduced was in the separation of the monoamino-acids and proline from the bases and dicarboxylic amino-acids. For this separation use was made of the butyl alcohol extraction procedure of Dakin [1918; 1920] which has proved so successful when applied by that worker to acid hydrolysates of proteins. The results of applying this procedure to the present case, however, were in keeping with the experience of Hunter [1925], who found that a sharp separation could not be obtained with enzymic digests of proteins containing peptide material. It is indeed hardly surprising that the butyl alcohol extraction method did not follow the same course and was not as complete and clear cut with the present material as with a protein hydrolysate, because of the presence, in addition to amino-acids, of a host of other organic and inorganic compounds. There is, for example, little doubt that acid-hydrolysable peptides were present, as Vickery has shown to be the case with alfalfa juice. Nevertheless, in spite of these circumstances, the method was of value and made it possible to isolate and identify tyrosine, *l*-leucine, *d*-*iso*leucine, *d*-valine, *d*-arginine, *i*-proline. The general scheme of fractionation is outlined in Table 2.

Table 2. *Outline of fractionation procedure*



Every attempt to arrive at the composition of a complex organic mixture of biological origin such as a solution of chemical entities constituting a plant juice requires in the first instance much preliminary pioneering work to determine what substances and groups of substances are actually present. In the initial stages of the present study an attempt was made to keep an audit of the nitrogen separation, but many of the fractions obtained proved on further investigation to be so intractable that this had soon to be abandoned.

EXPERIMENTAL

A sample of serum solids, supplied by the Central Rubber Station, Buitenzorg, Java, and prepared by evaporating serum obtained by the coagulation of fresh latex with acetic acid, was used in some preliminary experiments. It was found that, although originally completely water-soluble, an appreciable amount had become insoluble during evaporation and storage. This insoluble material was isolated by filtration, washed with hot water and dried. The resulting hard, dark brown material had no melting point and was found to be protein in nature with 2·15 % ash. Repeated solution in NaOH and precipitation with acid gave a fairly white product. It will thus be seen that originally this protein was a normal soluble constituent of rubber latex, and that, while it was not precipitated by acetic acid (in the cold) during the coagulation of latex, subsequent heating had denatured it.

According to the view of Belgrave [1925] and of Belgrave & Bishop [1923] the non-protein nitrogenous compounds in latex are destined for the synthesis of proteins or are their decomposition products. If this be true then an analysis of the protein for constituent amino-acids should give helpful information as to nitrogenous substances which might be expected to occur in the free state in latex serum. With this object in view a cursory examination of the protein was made.

The protein (100 g.) was hydrolysed with six times its weight of H_2SO_4 (38 %) for 24 hr. The mineral acid was removed by adding baryta to *p*H 3 and the solution of amino-acids was concentrated to 350 ml., when tyrosine began to crystallize out. The solution was then brought to *p*H 5·5 with KOH and concentrated to 200 ml. After 18 hr. at 0° the separated material was filtered off. On repeated crystallization from hot water, this gave 2·5 g. of pure tyrosine.

The concentrated solution of amino-acids was then subjected to a continuous butyl alcohol extraction [Dakin, 1918; 1920]. The fraction separating from the butyl alcohol (monoamino-monocarboxylic acids, aliphatic and aromatic) weighed 31 g. From this mixture glycine was isolated as the ethyl ester hydrochloride: M.P. 144°.

The fraction soluble in butyl alcohol (proline and hydroxyproline) weighed 5·14 g. Some difficulty was experienced in isolating proline as hydantoin but evidences of its presence were found. The fraction not extracted by butyl alcohol was, after removal of the solvent, passed through the Foreman [1914] procedure for the separation of the dicarboxylic acids. In this way 11·4 g. of copper aspartate and 1·75 g. of glutamic acid hydrochloride (M.P. 196–197°) were obtained. The base fraction obtained from the Foreman filtrate by precipitation with phosphotungstic acid was examined by the Kossel procedure as given by Plimmer [1917] and although, for various reasons, the analysis was not completed, evidence of the presence of arginine, histidine and lysine was obtained.

From the cursory examination of the protein described above it will be seen that it is quite similar to most plant proteins, and, on the hypothesis of Belgrave, gives hints as to the possible amino-acids present in the serum of *Hevea brasiliensis.*

The actual work on serum solids was done on a quantity of serum solution (kindly furnished by the Naugatuck Chemical Company) obtained from latex preserved with ammonia and containing sodium alginate, which had been used to cause separation of the rubber by creaming. An attempt was made to evaporate this serum solution *in vacuo*, but the presence of sodium alginate caused violent frothing and it was necessary to concentrate from 55 gallons to 10 l. at atmospheric pressure and 75° with the aid of constant stirring and an electric fan to carry off the vapour. The time taken was 74 hr., and during the concentration about 2 kg. of rubber separated in small flocks, which were removed, together with coagulated protein, by skimming. Towards the end of the operation magnesium oxide was added to remove ammonia. Finally, the cooled concentrate was diluted with alcohol to precipitate the residual rubber and protein, together with the major part of the alginic acid, which could then be filtered off. To the filtrate were added 1200 g. normal lead acetate to remove proteoses and inorganic acids having insoluble lead salts and also to help clarify the solution under investigation. Not much precipitation occurred, however, until the solution was diluted with water. The precipitate was filtered, washed and the filtrate and washings saturated with H_2S to remove lead. The clarified solution was then concentrated *in vacuo* at 30° in the presence of charcoal, filtered and made up to a volume of 10 l. Of this 3800 ml. were taken for further investigation, and the results of a preliminary analysis by standard methods are given in Table 3.

Table 3

	g. in 3800 ml.	% of total N
Total N	124·6	100
Ammonia-N	17·4	14
Amide-N	4·1	3·3
Amino-N	23·4	18·5
Other N	80·0	64·1
Solids	2180·0	—
Ash	286·0	—

Precipitation with Neuberg's reagent

The solution was mechanically stirred and 40 % Na_2CO_3 added until the liquid was acid to phenolphthalein but alkaline to litmus. After standing 2 hr., 21 g. of inorganic solids were removed by filtration. To the filtrate were added in small alternate quantities 1900 g. Na_2CO_3 (in 10 % solution) and 4750 g. mercuric acetate (in 25 % solution) until red mercuric oxide began to separate. After a day at 4–5° the voluminous precipitate was filtered off and washed with 80 % alcohol. The filtrate and washings were combined and will be referred to later as the 'Neuberg filtrate'.

The Neuberg precipitate was suspended in as small a quantity of water as possible and treated with H_2S. When the liberation of the nitrogenous material was complete, the solution was kept at 70° for a number of hours to granulate the mercuric sulphide. After filtering and washing with hot water, the filtrate and washings were concentrated *in vacuo* at 35° and the volume made up to 2 l. The analysis at this stage is given in Table 4.

On comparing Tables 3 and 4, it will be seen that the Neuberg precipitate has carried down 82 % of the total N of the original solution, and that the amide-N has disappeared, probably by hydrolysis in the alkaline medium, thus augmenting the yield of ammonia-N.

Table 4

	g. in 2 l. ml.	% of total N
Total N	101·9	100
Ammonia-N	19·0	18·65
Amide-N	None	None
Amino-N	23·9	23·5
Other N	59·0	57·9
Solids	1094·2	—
Ash	211·8	—

H_2SO_4 was next added to the above-mentioned solution obtained from the Neuberg precipitate until the concentration of the acid was 5%; this was followed by 25% aqueous phosphotungstic acid containing 5% H_2SO_4, added in small quantities at a time with stirring, until a drop of the supernatant liquid gave a white precipitate with a drop of baryta. The reaction mixture was kept at 5° for 50 hr., filtered and the precipitate washed well with 5% H_2SO_4. The procedure up to this point has separated the original solution of serum solids into three fractions (cf. Table 1):

(*a*) 'Neuberg filtrate': a fraction containing nitrogenous substances not precipitated by mercuric acetate-sodium carbonate.

(*b*) 'Phosphotungstic acid precipitate': a fraction containing nitrogenous substances precipitated by mercuric acetate-sodium carbonate and by phosphotungstic acid, viz. purines, diamino-acids.

(*c*) 'Phosphotungstic acid filtrate': a fraction containing nitrogenous bodies precipitated by mercuric acetate-sodium carbonate but not by phosphotungstic acid, viz. monoamino-acids.

Neuberg filtrate

The Neuberg precipitate having been removed, the filtrate was diluted with an equal volume of 95% alcohol (18 l.) and kept for 24 hr., after which some mercuric acetate was removed as a co-ordination compound with mercuric oxide [Berthelot, 1883]. The filtrate was now acidified with acetic acid and H_2S passed in to remove traces of mercury. After filtration the combined filtrate and washings were concentrated *in vacuo* at 35° to 8 l. Since 1900 g. of Na_2CO_3 had been introduced into this solution during the Neuberg precipitation and since the sodium was now present in the form of both acetate and carbonate, the following method was used to remove the sodium ion as chloride and the acetate ion as acetic acid.

The solution was first acidified to *p*H 3 with HCl. This caused the separation of a large quantity of NaCl and liberated all the acetic acid. The salt was removed by filtration and the filtrate concentrated until sufficient NaCl had separated to make the vacuum distillation difficult. The salt was again removed and distillation continued. In this way practically all the acetic acid was removed and the volume of the solution reduced to 2·5 l. At this point 2·5 l. of 95% alcohol were added to the concentrate, whereby 500 g. of NaCl and KCl were removed. The clear filtrate was reduced *in vacuo* to 1 l. and an equal volume of alcohol added. This threw down much quebrachitol and some inorganic material. These were filtered off and washed with alcohol. The filtrate and washings were concentrated to remove all alcohol, and then submitted to the continuous butyl alcohol extraction of Dakin [1918; 1920] for 200 hr. to remove any proline that might have escaped precipitation by the Neuberg reagent. It was found that the butyl alcohol extracted a fair amount of material, including most of the pigment, from the aqueous solution, and that on removing the solvent the resulting thick paste was very soluble in water. 5 g. of this paste were treated for proline by the method of Dakin [1920]. To the solution in 50 ml. of water 8 g. of potassium cyanate were added and the whole evaporated to dryness on the water bath. The mass was taken up with water and acidified with H_2SO_4 until acid to Congo red (pH 3). The solution was then extracted with ether for 7 hr. in a continuous extractor [Dakin, 1920] to remove monoamino-acids, if present. When the extraction was complete 1/5 vol. of H_2SO_4 (50 %) was added and the solution heated on the water bath for 2 hr. to convert the proline into the hydantoin. The solution was again extracted with ether for 50 hr. when there were obtained 26 mg. of substance which melted at 141–144°. The melting-point of *i*-proline hydantoin is given by Dakin [1920] as 143°. (Found by Folin's colorimetric method [1925]: N, 21·1 %; $C_6H_8O_2N_2$ requires N, 20·0 %.) The small yield did not permit of further confirmatory evidence being obtained. Small-scale experiments using mercuric chloride gave no evidence for the presence of betaines in this fraction.

Phosphotungstic acid precipitate

The wet precipitate was suspended in 3 l. of water and decomposed by adding baryta until alkaline to litmus. The magma was kept warm for 3 hr., filtered, the barium phosphotungstate well washed with hot water and the filtrate and washing reduced *in vacuo* to 3 l. The last trace of barium was now removed in the usual way and the final volume adjusted to 2 l. This is the 'purine and diamino-acid fraction', which analysed as follows:

	g.
Total solids	203·6
Ash	98·4
Total N	18·6

The fraction was treated with $AgNO_3$ (120 g.), which threw down a very dense canary yellow precipitate of purine-silver nitrate complexes. This was filtered, washed with hot water and decomposed with H_2S. The filtrate on evaporation gave 91 g. of a mixture of yellow crystals and a dark-brown syrup having an ash content of 65·1 %, the residue being almost pure tungstic oxide. The mixture contained 2·8 g. N, but attempts to separate the purine bases from it were unsuccessful.

The histidine fraction. The above-mentioned filtrate, which might contain the three bases, was submitted to the usual Kossel procedure for their isolation. Baryta was added until a drop of supernatant liquid no longer gave a white precipitate with a reagent made by adding ammonia to a 10 % silver nitrate solution until the silver just dissolves. The precipitate was kept in the cold for several hours before filtering and washing with cold water. It was then decomposed with H_2S and the clarified filtrate taken to dryness *in vacuo*; 1·5 g. of a light lemon-yellow coloured solid were obtained, which gave none of the usual reactions for histidine. It is inferred, therefore, that the latex serum does not contain this base.

The arginine fraction. The base solution was next saturated with baryta at 40°. After several hours the arginine-silver complex was separated by filtration, decomposed in the usual way and concentrated under reduced pressure. The solid material thus obtained weighed 4 g., and the ninhydrin test showed that α-amino-N was present. Arginine was obtained from it as flavianate by the method of Vickery [1926] and for further characterization was converted into the carbonate by the method of Pratt [1926]. The yield of white crystalline material was 1·42 g.: 0·2410 g. when heated at 105° for 4 hr. lost 0·104 g., showing the presence of 0·5 mol. of water of crystallization (no previous reference in the literature). For the air-dried material: C = 37·6; H = 6·66 %. $C_7H_{14}O_4N_4$, 0·5 H_2O requires C, 36·95; H, 6·61 %. For the anhydrous material: N = 25·15 %. $C_7H_{14}O_4N_4$ requires N, 25·7 %. The silver salt of arginine was made from 0·2 g. by adding sufficient HNO_3 to form the mononitrate and then adding the equivalent of silver nitrate. On diluting with alcohol, then with ether and keeping the mixture in the dark, white needles separated slowly (M.P. 181–183° with decomposition; Riesser [1906] gives 183°). Found: Ag, 26·64 %. $C_6H_{14}N_4O_2.HNO_3.AgNO_3$ requires Ag, 26·15 %. The picrate melted at 203–205° (Mulliken [1916] 205–206°) and the anhydrous copper salt at 238–240° (Riesser [1906] 232–234°). A solution made by dissolving 1 % of the carbonate in 5 % HCl showed in a 1 dm. tube $[\alpha]_D^{20} + 9{\cdot}1°$.

From the above evidence it is certain that the compound isolated was *d*-arginine and that it therefore occurs as such in the original solution of serum solids.

The lysine fraction. Silver and barium were completely removed from the arginine filtrate and the clarified solution concentrated to 510 ml. The mercuric chloride-baryta treatment of Winterstein [1904] gave 5 g. of a dark-brown hygroscopic mass from which it was not found possible to isolate lysine as its picrate. Other attempts to obtain some evidence for the presence of this base also failed.

Phosphotungstic acid filtrate

The amino-acid fraction which was precipitated by Neuberg's reagent but not by phosphotungstic acid was submitted to continuous extraction with butyl alcohol to give:

(*a*) A fraction soluble in butyl alcohol.

(*b*) A monoamino-acid fraction insoluble in, but extracted by, butyl alcohol.

(*c*) A monoaminodicarboxylic acid fraction not extracted by butyl alcohol.

It must be stated that this extraction was by no means as clear cut as in the case of hydrolysed proteins for which it was primarily designed, but even so a sufficient separation of material was obtained to lead to the isolation of a number of amino-acids.

The extraction was continued for 200 hr., the butyl alcohol being changed every 24 hr. At the end of each day the butyl alcohol was filtered to remove fraction (*a*) (see above) and the residue washed with fresh alcohol. The filtrates were collected, the alcohol removed *in vacuo* and the residue taken up in water (fraction (*b*)). The aqueous solution of unextracted material was evaporated *in vacuo* to remove butyl alcohol and some acetic acid, and there was thus obtained a thick golden-yellow paste (fraction (*c*)).

Fraction (*a*). The solution (500 ml.) on standing deposited 4·4 g. of nearly colourless crystals which gave a strong Millon reaction indicating the presence of tyrosine. The material was suspended in 150 ml. of 95 % alcohol and boiled, water being added slowly until no more of the suspension went into solution. The residue was filtered off and washed with alcohol. The dried material (0·69 g.) was dissolved in hot water, treated with norit (0·1 g.), filtered and concentrated on the water bath to incipient crystallization. On cooling, characteristic crystals of tyrosine separated and were filtered off (0·41 g.). The mother liquor on further concentration yielded an additional 0·11 g. The Millon test was positive. (Found: C, 59·9; H, 6·0; N, 7·69 %. $C_9H_{11}O_3N$ requires C, 59·7; H, 6·1; N, 7·73 %.) On standing, the solution from which the crude tyrosine had separated deposited thin lustrous crystals. These were filtered off and washed with cold alcohol (1·62 g.). Millon's test was negative, showing the absence of tyrosine. Their greasy feel, the fact that they were moistened by water only with difficulty, and especially their analysis, suggested that they were leucine (found: N, 10·68; amino-N, 10·94 %; mol. wt. (freezing point of water) 126·7; $C_6H_{13}O_2N$ requires N, 10·7; amino-N, 10·7 %; mol. wt. 131), but the specific rotation in water, $[\alpha]_D^{20} -13{\cdot}6°$, was too low and in 20 % HCl was too high $[\alpha]_D^{20} +27{\cdot}1°$, the recorded values in the literature being $-10{\cdot}42°$ and $+15{\cdot}53°$ respectively. Further investigation showed that the product was a mixture, and *l*-leucine was identified as its phenylhydantoin, M.P. 119–120°, and *d-iso*leucine as its benzoyl derivative, M.P. 116°.

The mother liquor from the above separation was concentrated to a syrup and kept at 0° for 24 hr. A mass of crystalline material was deposited which was filtered off and washed with ethyl acetate. The product (6·73 g.) was recrystallized from hot water to give ill-defined colourless platelets (2 g.) which were subsequently shown to be a mixture of *l*-leucine and *d-iso*leucine. The final mother liquor was taken to dryness (473 g.) dissolved in the minimum quantity of water and treated with much alcohol. On standing, another crop of crystalline material (2·6 g.) was obtained. This was filtered off, dissolved in water and precipitated by adding a large volume of alcohol. The product was *d*-valine. (Found: C, 50·4; H, 9·45; N, 11·88 %. $C_5H_{11}O_2N$ requires C, 51·3; H, 9·40; N, 11·96 %.) The specific rotation in water was $[\alpha]_D^{20} +6{\cdot}3°$ and in 20 % HCl $[\alpha]_D^{20} +28{\cdot}6°$, while the phenylcarbamido-acid melted at 154–153° and the phenylhydantoin at 129–130·5°, values that are in keeping with those recorded in the literature.

Fraction (*b*). This weighed 7·8 g. and contained 30 % of ash. 2 g. of the material were dissolved in 10 ml. of *N* NaOH and to the cooled solution an equivalent of phenylisocyanate was added. The mixture was shaken, with cooling, until the odour of isocyanate had disappeared. After filtration the phenylcarbamido-acids in the filtrate were precipitated by adding 10 ml. of *N* HCl. On recrystallization 1 g. of the phenylcarbamido derivative of *iso*leucine, M.P. 119–120°, was obtained. The hydantoin obtained by dehydration with HCl melted at 80°.

Fraction (*c*). After standing for about two months this fraction showed no signs of crystallization; it was therefore saturated with gaseous HCl, which precipitated much sodium and potassium chlorides. These were removed by filtration, and the filtrate set aside at 4–5° for 5 weeks, but no glutamic acid separated. A similar result was obtained in attempting to isolate glycine ethyl ester. The material was therefore collected, water removed, the residue covered with 4 vol. of 95 % alcohol and saturated with dry HCl to precipitate most of the inorganic material (150 g.) and to bring the remainder into solution (as ethyl ester). After filtration, the alcohol and water were then removed *in vacuo*, 4 vol. of absolute alcohol were added, and the mixture again saturated with gaseous HCl.

The esterified solution was evaporated almost to dryness under reduced pressure and the residue dissolved in the smallest quantity of water. The esters were liberated with NaOH and extracted with ether after saturation of the liberated ester solution with K_2CO_3. When the greater part of the dry ether was evaporated, 6 vol. of distilled water were added and the solution refluxed for 6 hr. to hydrolyse the esters to the free amino-acids and ethyl alcohol. Ordinary distillation removed most of the alcohol and evaporation under reduced pressure left a solid residue weighing 38 g. and also gave a turbid distillate from which were obtained 2 ml. of a pungent-smelling oil. This oil was set aside to be investigated at some future date. The solid residue was dissolved in as little hot water as possible and decolorized with animal charcoal. To the clear filtrate 500 ml. of acetone were added, which caused the separation in two crops of 16 g. of material, M.P. 259–263°. Appropriate tests showed that this contained no glutamic acid, but that the ninhydrin reaction was positive. Negative Millon and positive xanthoproteic tests indicated the presence of phenylalanine. It was found, after a great deal of experimentation, that this material was not completely soluble in cold glacial acetic acid; 4 g. were accordingly suspended in cold glacial acetic acid and filtered rapidly, the residue being washed repeatedly with small quantities of the acid. In this way 1·04 g. of a pure white crystalline substance were obtained. Analysis of the substance, and of its insoluble copper salt, showed that it was *l*-aspartic acid. (Found: C, 36·4; H, 5·27; N, 10·58%. $C_4H_7O_4N$ requires C, 36·1; H, 5·26; N, 10·52%.) The specific rotation in 1% aqueous solution was $[\alpha]^{20}_{D}-4{\cdot}1^\circ$. The copper salt contained 23·0% Cu; $C_4H_5O_4NCu,4{\cdot}5H_2O$ requires Cu 23·05%.

SUMMARY

The following amino-acids are shown to occur in the serum of the latex of *Hevea brasiliensis*, all except the last having been actually isolated from it: tyrosine, *l*-leucine, *d*-*iso*leucine, *d*-valine, *d*-arginine, *l*-aspartic acid, *i*-proline, phenylalanine. A cursory examination of the amino-acid composition of the heat-coagulable protein of latex has been made. A tabular summary is given of the results obtained by various workers to date on the amino-acid composition of latex and latex proteins.

The authors are deeply indebted to Prof. A. C. Chibnall for assistance in revising the manuscript of this paper.

REFERENCES

Altman (1935). *Arch. Rubbercult. Ned.-Ind.* **23**, 239. (Reprinted *Rubb. Chem. and Technol.* **13**, 505 (1940).)
Belgrave (1925, 1). *Malay. agric. J.* **13**, 154.
—— (1925, 2). *Malay. agric. J.* **13**, 367.
—— & Bishop (1923). *Malay. agric. J.* **11**, 371.
Berthelot (1883). *Ann. Chim. (Phys.)*, **29**, 392.
Dakin (1918). *Biochem. J.* **12**, 290.
—— (1920). *J. biol. Chem.* **44**, 499.
Folin (1925). Laboratory Manual of Biological Chemistry, p. 131.
Foreman (1914). *Biochem. J.* **8**, 463.
Hunter (1925). *Trans. roy. soc. Can.* **19**, (V), 1.
McGavack & Rumbold (1934). *Industr. Engng Chem.* **26**, 789.
Midgley, Henne & Renoll (1937). *J. Amer. chem. Soc.* **59**, 2501.
Mulliken (1916). Identification of Pure Organic Compounds. London: Chapman & Hall.
Neuberg & Kerb (1912). *Biochem. Z.* **40**, 498.
Plimmer (1917). The Chemical Constitution of the Proteins, Part 1. London.
Pratt (1926). *J. biol. Chem.* **67**, 351.
Riesser (1906). *Hoppe-Seyl. Z.* **49**, 217.
Tristram (1940). *Biochem. J.* **34**, 301; cf. Tristram (1941). *Biochem. J.* **35**, 413.
Vickery (1924). *J. biol. Chem.* **60**, 647; **61**, 117.
—— (1925). *J. biol. Chem.* **65**, 81, 91, 657.
—— (1926). *J. biol. Chem.* **71**, 303.
—— and Leavenworth (1925). *J. biol. Chem.* **63**, 579.
Winterstein (1904). *Hoppe-Seyl. Z.* **45**, 77.
Whitby, Dolid & Yorston (1926). *J. chem. Soc.* p. 1448.
Zwikker (1914). *Pharm. Weekblad*, **51**, 21; *Chem. Abstr.* **8**, 841.

67. A COLORIMETRIC METHOD FOR THE DETERMINATION OF CITRULLINE[1]

By ALLAN GODFREY GORNALL and ANDREW HUNTER
From the Department of Pathological Chemistry, University of Toronto

(*Received 31 May 1941*)

In the course of experiments on the mechanism of urea formation, we came to feel the need of a convenient method for the determination of small quantities of citrulline. The carbamido-diacetyl reaction described by Fearon [1939] appeared to offer a prospect in that direction. Fearon himself had utilized it in an approximate determination of the citrulline content of trypsin-digested casein, but in general his observations were qualitative. We are indebted to him for permitting, and indeed encouraging, us to explore independently the possibilities of his test as the basis of a quantitative method.

The reaction consists in the development of a red (in the case of urea a yellow) colour, when the reacting substance is heated with diacetyl monoxime in acid solution and the product subsequently oxidized with potassium persulphate. It is not specific for citrulline, being given apparently by all compounds of the general formula $R.\mathrm{NH.CO.NH_2}$ and by some of the formula $R_1.\mathrm{NH.CO.NH}.R_2$. To Fearon's list of substances tested and found to react positively we are able to add the α-carbamido derivatives of propionic, *n*-butyric, *n*-valeric, *iso*butylacetic, δ-guanidinovaleric acid and δ-aminovaleric acids, β-carbamidopropionic acid, ϵ-carbamidohexoic acid and 3-carbamido-2-piperidone [Hunter, 1938]. Among known tissue constituents, other than citrulline itself, the only ones that give the test are urea, allantoin, proteins and such higher protein derivatives as peptone and gelatin.

In adapting the reaction to the quantitative determination of citrulline we have employed the method of photoelectric colorimetry, using the instrument of Evelyn [1936]. This possesses the advantage over most similar forms of apparatus (of particular importance for the present application) that the reaction is carried out, and the colour developed, in the same special test tubes, which serve as the absorption cells of the colorimeter. The procedure recommended could doubtless be adapted to any other photoelectric colorimeter. For visual colorimetry it is hardly suitable.

Lacking facilities for a precise spectrophotometric study of the coloured reaction mixture, we had to be guided, in the choice of a suitable light filter, by an approximate procedure, in which the photocolorimeter itself is used as a spectrophotometer. A spectrogram of the red solution (for the preparation of which we are indebted to Dr Ireton and Dr Allin of the Department of Physics, University of Toronto) revealed an absorption band in the green and blue, extending roughly from 460 to 520 mμ. Following this indication, we applied the analytical procedure, presently to be described, to six 7 ml. samples of a 0·001 % citrulline solution, using with successive samples each in turn of the six Rubicon filters numbered (in correspondence with the dominant wave-length of the transmitted light) from 420 to 565 mμ. The six values for photometric density thus obtained were plotted against the filter numbers or dominant wave-lengths. The resulting curve suggested that the maximum of absorption occurs at or near 475 mμ. A filter ideal for that wave-length not being immediately procurable, No. 490 (transmission limits 465 to 530 mμ) was adopted as the best available compromise.

---

[1] The method was described, and a preliminary account was given of some results already obtained with it, at a meeting of the Royal Society of Canada held at Kingston, Ontario, on 21 May 1941.

It may here be mentioned that we constructed similar rough absorption curves for urea, allantoin, methyl- and phenyl-urea, α- and β-carbamidopropionic acids, ϵ-carbamidohexoic acid and 3-carbamido-2-piperidone. For the four last of these the curves were to all appearance identical, both in form and position, with the curve for citrulline. From that pattern methyl- and phenylurea showed only minor deviations. The curve for urea, as was to be expected, was conspicuously different, its peak falling in the neighbourhood of 450 mμ. Allantoin yielded a curve differing little from that of urea.

In order to obtain consistent results in the quantitative application of Fearon's test it was found necessary to define very rigidly both the proportions of the reagents and the conditions under which the colour is developed. The technique we are about to describe, adopted after many exploratory experiments, not only ensures the reproducibility of the results, but enhances considerably the sensitivity of the reaction. It permits the estimation of citrulline in any concentration down to (for pure solutions) 0·2 or 0·3 mg./100 ml., with an error which, except at the very lowest concentrations, does not exceed 2 % (see Table 2).

Description of the method

The following description takes for granted an acquaintance with the theory and the general operational technique of the Evelyn photoelectric colorimeter [Evelyn, 1936; Dann & Evelyn, 1938] and omits mention of details common to all methods in which that instrument is employed.

A constant-level water bath is provided with a cover and a perforated false bottom. The cover is pierced with a number of round holes, of a diameter (about 22 mm.) exactly fitting the absorption tubes of the colorimeter. The false bottom is fixed at such a level that the vertical distance between it and the cover is between 70 and 90 mm. The water level is adjusted so that the height of water above the false bottom is between 45 and 50 mm. The dimensions specified are such that during the period of heating only that part of the tube will be immersed which is charged with the reacting mixture, while half at least of its length will project above the cover. The objects sought are the standardization of the heating and the avoidance, as far as possible, of concentration through evaporation. The second of these objects requires that the projecting part of the tube should be kept as cool as possible. All the holes in the cover should therefore either be occupied by tubes or covered by small watch-glasses. It is of advantage also to have a number of holes in the wall of the bath below the cover, so that escaping steam will be directed away from the tubes.

While the water in the bath is kept gently, but steadily, boiling, a measured volume of the citrulline-containing solution is transferred to one of the special absorption test-tubes of the colorimeter. The volume should be such as to contain about 0·05–0·07 mg. of citrulline—in no case less than 0·02 or more than 0·12 mg. Water is added to make a total volume of exactly 7 ml. Alternatively the solution may first be diluted to a concentration of 0·7–1 mg./100 ml., 7 ml. being then measured into the absorption tube. If the concentration of citrulline is quite unknown to begin with, one or more random trials will usually indicate the quantity or the dilution proper for a precise determination.

To the citrulline solution there are added (1) 4 ml. of conc. HCl (sp.gr. 1·18–1·19) and (2) 0·5 ml. of a 3 % solution of diacetyl monoxime. The reactants having been mixed, the tube is placed in the bath, and a 1 in. funnel is set in its mouth to act as a condenser. The tube is left in the boiling water for *exactly* 9 min. It is then removed, wiped dry and allowed to cool in the air for 6 min. This will reduce its temperature to about 65°. If citrulline is present, the mixture will already show a pink colour. To develop this more fully, there is now added one drop of 1 % potassium persulphate solution. The tube is thereupon placed in position in the colorimeter (already equipped with the appropriate filter, No. 490, and adjusted to the reagent-blank rest point), and the behaviour of the galvanometer spot is observed. As the colour deepens, the galvanometer reading, of course, falls. When it shows signs of becoming stationary, another drop of persulphate is added. If this produces a further fall, a third drop may be needed to make sure that the maximum attainable depth of colour and the minimum galvanometer reading have been reached. This is usually accomplished in 1–2 min. After a time the colour begins to fade, and the galvanometer reading to rise again. The lowest reading observed is the significant one. It is translated into terms of citrulline concentration by reference to a previously constructed calibration curve.

A calibration curve is necessary because, with the technique described, the logarithmic law of Lambert and Beer holds good only over a limited range of concentrations. The reason may be that the filter used is insufficiently selective, or that with the lower concentrations fading of the colour overtakes its full development. A curve for use with pure solutions may be constructed from the data recorded in Table 1. In practice only that part of the curve would ordinarily be used, which lies between galvanometer readings of 80 and 20 (the respective citrulline concentrations being 0·35 and 1·7 mg./100 ml.). The concentration read from the curve is of course that existing in the volume of 7 ml. directly analysed. From this the concentration in the original solution is readily calculated.

Table 1. *Calibration curve for pure solutions of citrulline*

ml. of stock citrulline solution (5 mg./100 ml.) in a total volume of 7 ml.	Final citrulline concentration (mg./100 ml.)	Galvanometer reading (average of two to three trials)
0	0	100 (R.P. = 66)
0·3	0·214	88·75
0·6	0·429	73·7
0·9	0·643	59·9
1·2	0·857	48·0
1·5	1·071	38·0
1·8	1·285	29·4
2·1	1·50	23·7
2·4	1·715	19·3
2·7	1·93	16·25
3·0	2·14	13·3
3·6	2·57	10·0

The time occupied by a single determination (from the addition of the reagents to the final reading of the galvanometer) is 16–17 min., and so simple are the manipulations required, that a series of determinations can be carried along in orderly progression at regular intervals of 3 min.

To the foregoing description of the method we may add here the following comments. With a given amount of citrulline the depth of colour obtained depends upon the proportions of HCl and diacetyl, upon the intensity and duration of the heating and, in a smaller measure, upon the length of the subsequent cooling period. In the procedure prescribed the reagents are applied in what have been found to be the optimal concentrations with respect to colour development. The period of hearing, on the other hand, is a compromise. If it is shortened, less colour is produced, and the sensitivity of the method is correspondingly diminished. If it is lengthened, colour production and sensitivity are increased; but the method then becomes rather tedious. A change in either direction, while necessitating the construction of another calibration curve, is not only permissible, but might in special circumstances be of advantage. The essential point is not that the reaction mixture be heated for 9 min., but that both the time and the manner of heating be always exactly the same. The prescribed time of cooling need not be quite so rigidly adhered to.

Accuracy of the method with pure solutions

In the preparation of the calibration curve the agreement between duplicate or triplicate runs on any one concentration of citrulline was always within 2 %. It was to be expected that in the analysis of pure solutions the results obtained would be equally good. This expectation is confirmed by the examples given in Table 2. Only occasionally does the error exceed the proportion named.

Table 2. *Analysis of pure solutions of citrulline of known concentration*

Concentration, mg./100 ml.	
Known	Found
0·236	0·22
0·59	0·58
0·67	0·67
0·75	0·73
0·75	0·75
0·826	0·83
1·00	1·00
1·00	0·99
1·00	1·02
1·46	1·46

Behaviour of reacting substances other than citrulline

In any practical application of the method, account has to be taken of certain difficulties, the first of which is the lack of specificity of the carbamido-diacetyl reaction. Among the substances which give this reaction there are at least three which may be expected to be present in practically every animal tissue or fluid: protein, urea and (except in human material) allantoin.

Protein and its higher derivatives occupy an exceptional position in that they yield, under the conditions prescribed, not only what appears to be a citrulline reaction, but also an additional violet colour (Liebermann reaction) partially or completely obscuring the other. Fearon discusses means of minimizing this effect in the qualitative test, but we have found no satisfactory way of preventing or compensating for it in the quantitative technique. Any solution to be analysed for free citrulline must therefore first be rendered protein-free. Among possible deproteinizing agents the most suitable is trichloroacetic acid. Uranyl acetate and tungstic acid may also be employed, although with the latter care must be taken to avoid such an excess, as would precipitate on the addition of the hydrochloric acid.

In order to evaluate the disturbing effects of urea and allantoin we analysed solutions of each in routine fashion, just as if they were solutions of citrulline. As a matter of interest (although of less immediate practical importance), we also treated in the same way solutions of the other carbamido compounds listed in Table 3. Each substance was tested in at least two concentrations. These are stated in the third column of the table, while in the fourth there is shown for each that concentration of citrulline to which, in carbamido content, it is equivalent. The fifth column records the citrulline concentrations which the method appeared to indicate, while in the last the 'citrulline' thus found is expressed as a percentage of the citrulline equivalent. For citrulline itself the figure in the last column is of course 100.

Table 3. *Behaviour of various substituted ureas in the proposed method for citrulline*

No.	Substance	Concentration tested mg./100 ml.	Equivalent concentration of citrulline mg./100 ml.	Apparent citrulline concentration found	
				mg./100 ml.	% of citrulline equivalent
1	Urea	0·4	1·17	0·43	36·7
		2·0	5·84	1·56	26·7
		4·0	11·67	1·86	15·9
2	Allantoin	10·0	11·1	0·86	7·7
		20·0	22·2	1·33	6·0
3	Methylurea	0·212	0·50	0·55	110·0
		0·846	2·00	2·00	100·0
4	Phenylurea	0·39	0·50	0·38	76·0
		0·777	1·00	0·75	75·0
5	α-Carbamidopropionic acid	10·0	13·3	0·46	3·5
		20·0	26·5	1·04	3·9
6	α-Carbamido-*n*-butyric acid	10·0	12·0	0·24	2·0
		20·0	24·0	0·51	2·1
7	α-Carbamido-*n*-valeric acid	10·0	10·9	0·24	2·2
		20·0	21·9	0·57	2·6
8	α-Carbamido*iso*hexoic acid	10·0	10·0	0·17	1·7
		20·0	20·0	0·42	2·1
9	α-Carbamido-δ-guanidino-*n*-valeric acid	10·0	8·1	0·19	2·3
		20·0	16·1	0·38	2·4
10	α-Carbamido-δ-amino-*n*-valeric acid	10·0	10·0	0·18	1·8
		20·0	20·0	0·38	1·9
11	β-Carbamidopropionic acid	0·377	0·50	0·375	75·0
		0·754	1·00	0·775	77·5
12	δ-Carbamido-α-amino-*n*-valeric acid (citrulline)	—	—	—	100·0
13	ε-Carbamido-*n*-hexoic acid	0·50	0·50	0·56	112·0
		1·00	1·00	1·11	111·0
14	3-Carbamido-2-piperidone	10·0	11·2	0·75	6·7
		20·0	22·3	1·56	7·0

The reactivity of allantoin is seen at once to be of a very low order. It seems unlikely that in conventionally prepared blood or tissue filtrates one would ever encounter an allantoin concentration as great as 1 mg./100 ml. Table 3 shows that this would be equivalent to 1·11 mg. of citrulline, but would yield, over the spectral wave-band employed, only 7·7 % as much colour—would simulate, that is to say, a citrulline concentration of only 0·086 mg./100 ml. In the uses to which we have hitherto put the method, this would be a negligible quantity. At the same time it must be recognized that substantial concentrations of allantoin, such for example as might occur in urine, would seriously complicate the application of the method.

The possible influence of urea is more important. A 1:10 protein-free filtrate of normal blood would contain about 3 mg./100 ml. of urea. From the data in Table 3 it may be estimated, by interpolation, that this would give about 20% as much colour as the equivalent amount (8·76 mg.) of citrulline. In the complete absence, therefore, of citrulline itself, the filtrate would show an apparent citrulline content of as much as 1·75 mg./100 ml. Similarly it may be calculated that a saline tissue extract containing, when diluted for a citrulline determination, 1 mg./100 ml. each of citrulline and urea (a combination such as has frequently arisen in our experiments) would yield, by the prescribed method, a citrulline value (1·95) almost double the true one. Since the urea colour is different from the citrulline one, the difficulty could perhaps be met by making two measurements of photometric density, each with a different, appropriate and sufficiently selective filter; but it is simpler to destroy the urea with urease, and this expedient we have accordingly adopted as a necessary preliminary to the determination of citrulline in any biological fluid. The destruction of urea can of course be conducted as a step in its manometric determination [Krebs & Henseleit, 1932]. Afterwards the urease can be removed, along with pre-existing proteins, by any suitable reagent.

Urea and allantoin are the only substances in Table 3 which come under consideration as regular tissue constituents. The others (citrulline itself excepted) are of interest only from a theoretical point of view. Six of them (Nos. 3, 4, 5, 11, 13 and 14) have been shown to yield, in the diacetyl reaction, products with absorption spectra differing little, if at all, from that of the citrulline product. As far as visual observation goes, the colour given by the remaining five (Nos. 6–10) is of exactly the same reddish shade. For all of these therefore (but not for urea or allantoin) the figures in the last column of Table 3 indicate the relative intensity with which the carbamido group reacts in the various combinations represented.

Regarded from this point of view the data show that, as a test for substituted ureas, the carbamido-diacetyl reaction varies in sensitivity with the character of the substituting group. Thus methylurea gives about the same depth of colour as an equivalent quantity of citrulline, but phenylurea gives only three-fourths as much, and piperidonylurea only one-fifteenth. Of particular interest are the results within the special group of the carbamido-acids, to which citrulline itself belongs. Here the intensity of the reaction appears to depend directly upon the distance separating the urea residue from the carboxyl. With α-carbamido-acids the reaction is feeble, and is little, if at all, affected by either the length of the fatty acid chain or the presence of such other substituents as the amino- or guanidino-group. The β-acid gives about 25 times more colour than the average α-acid, but not so much as the δ-acid, citrulline. The reactivity of the latter is surpassed, in turn, by the ε-acid. As one special example of these relations, citrulline is seen to react more than 50 times as intensely as its isomer, α-carbamido-δ-amino-*n*-valeric acid (No. 10).

Application of the method in tissue analysis

The determination of citrulline in biological material (in blood, for example, or in tissue extracts) requires, as has been shown, the prior removal of urea and proteins; but, even with this accomplished, other complicating factors have still to be dealt with. In the first place most urea- and protein-free filtrates, devoid though they may be of citrulline, still contain a trace of material giving the carbamido reaction. This trace may be too small to give a visible pink colour, yet large enough to simulate in the photocolorimeter a not entirely negligible content of citrulline. If this were all, the effect might be allowed for by deducting from all results with any particular material a constant 'blank', determined on similar material known to contain no citrulline. A more serious complication is, that tissue filtrates contain also something, which modifies in a peculiar way the development of the colour from any citrulline actually present. The effect is, that with low concentrations of citrulline colour development is greater, with high concentrations less, than in pure solutions. Under these circumstances the calibration curve already described might still give reasonably correct results for certain medium concentrations, but for all others it would be seriously misleading. It becomes necessary therefore to construct, for each practical application of the method, a special calibration curve.

The general procedure to be followed may be exemplified by describing in detail that application with which we have been most immediately concerned—the determination of citrulline, along with urea, in a saline medium containing the products derived from the metabolism of tissue slices. For the urea determination, to be carried out according to Krebs & Henseleit [1932], 3 ml. of the medium, it will be assumed, have been transferred to a suitable manometer vessel, and have then been treated with 0·3 ml. of acetate buffer (*p*H 5·0) and 0·3 ml. of urease solution. The manometer readings having been taken, the whole mixture is washed into a 15 ml. flask to a volume of about 12 ml. To this there is added 1 ml. of 50 % trichloroacetic acid, whereupon the flask is heated for 2–3 min. in boiling water. After having been cooled, the flask is filled to the mark with water, its contents are mixed and it is kept for half an hour. The contents are then filtered, and of the filtrate 7 ml. (or some suitable smaller volume diluted to 7 ml.) are taken for analysis.

The analysis is carried out in the manner already described, except that galvanometer readings are referred to a special calibration curve. In the case under consideration the preparation of this special curve is simplified by the fact that the blank, and also the materials which disturb the development of the colour, are derived exclusively from the added urease. The data for the curve, which are given in Table 4, were accordingly obtained as follows. Into a series of 15 ml. volumetric flasks were measured the stated volumes of a solution containing in 100 ml. 14·6 mg. of citrulline. To each were added 0·3 ml. of acetate buffer and 0·3 ml. of the urease solution employed in the determination. The mixtures were then diluted to about 12 ml., deproteinized, brought to volume

Table 4. *Calibration curve for urease filtrates*

ml. of stock citrulline solution (14·6 mg./100 ml.) in a total volume of 15 ml.	Final citrulline concentration (mg./100 ml.)	Galvanometer reading
0	0	100 (R.P. = 75)
0·25	0·243	73·5
0·50	0·487	60·5
0·75	0·73	49·5
1·00	0·973	40·5
1·25	1·216	33
1·50	1·460	26·5
1·75	1·703	21·75
2·00	1·946	18

and filtered as already described. From the first filtrate, containing no citrulline and serving therefore as the blank control, 7 ml. were transferred to an absorption test tube, and the colour was developed in the regular way. When the photometric density had, upon careful addition of the oxidizing reagent, reached its maximum, the galvanometer was quickly set to 100. The tube was thereupon removed, and the 'rest point' noted. The colour was then developed in the remaining tubes, galvanometer readings being taken with adjustment each time to the rest point already determined. In this way the blank was automatically allowed for in each of the readings, while the effect of other interfering factors was reflected in the course of the curve relating the galvanometer readings to the known concentrations of citrulline.

The curve given by the data of Table 4 starts at a higher level than that drawn from Table 1, but, falling more steeply, crosses it at a citrulline concentration of about 1 mg./100 ml. It is applicable, but applicable only, to all analyses carried out on 7 ml. samples of filtrates prepared, in the way prescribed, from similar material treated with a urease solution of identical origin and concentration. An ideal requirement is that the rest-point obtaining during any series of determinations should be identical with that found during the preparation of the calibration curve. In practice a difference of several points is admissible.

For any other special application of the method another calibration curve, constructed on the same principles, would have to be prepared.

With respect to the urease it is of advantage, naturally, to employ a preparation which, in the quantity requisite, gives the lowest blank and the least interference. A crude jack-bean extract is, from this point of view, rather unsuitable. We have found it best to use a urease powder prepared from jack-bean meal by the method of Van Slyke & Cullen [1914] but further purified by a second extraction and a second precipitation with acetone. A 10 % suspension of this powder is of ample potency, and yields (with 7 ml. of filtrate) a blank of only 0·2 mg./100 ml. It was such a preparation that was used in securing the data of Table 4.

To test the accuracy of the whole procedure we used it, exactly as described, in the analysis of known mixtures of urea and citrulline. The results, as shown in Table 5, were entirely satisfactory.

Table 5. *Analysis of citrulline-urea mixtures*

Mixtures analysed					Urea concentration (mg./100 ml.)		Citrulline concentration (mg./100 ml.)	
ml. of urea solution 27·3 mg./100 ml.	ml. of citrulline solution 16·5 mg./100 ml.	Water to ml.	ml. acetate buffer (*p*H 5·0)	ml. urease 10% solution	Present	Found	Present	Found
0·5	0·3	3	0·3	0·3	4·55	4·47	1·65	1·60
1·0	0·6	3	0·3	0·3	9·10	8·97	3·30	3·35
1·5	1·2	3	0·3	0·3	13·65	13·23	6·60	6·70
2·0	0·9	3	0·3	0·3	18·20	17·65	4·95	4·85

In conclusion it may be reported that the method has enabled us to demonstrate the production by liver slices of substantial quantities of citrulline from ornithine, and thus for the first time to establish citrulline as an actual product of tissue metabolism. A full account of the relevant observations is in process of preparation.

SUMMARY

The carbamido-diacetyl reaction of Fearon is made the basis of a method whereby, through the use of a photoelectric colorimeter, small quantities of citrulline may be estimated with an error not exceeding 2 %. Procedures are described for overcoming certain difficulties that arise in the application of the method to tissue analysis. Incidentally a comparison is made of the reactivities to Fearon's test of various carbamido-acids and other urea derivatives.

REFERENCES

Dann & Evelyn (1938). *Biochem. J.* **32**, 1008.
Evelyn (1936). *J. biol. Chem.* **115**, 63.
Fearon (1939). *Biochem. J.* **33**, 902.
Hunter (1938). *Biochem. J.* **32**, 826.
Krebs & Henseleit (1932). *Hoppe-Seyl. Z.* **210**, 33.
Van Slyke & Cullen (1914). *J. biol. Chem.* **19**, 211.

68. HEMICELLULOSE PREPARATION AND CHLORINATION CELLULOSE

By ISAAC ARTHUR PREECE

From the Chemistry Department, Heriot-Watt College, Edinburgh

(*Received 30 May 1941*)

It has recently been shown that the use of boiling alcoholic-NaOH pretreatment of wood as a preliminary to hemicellulose extraction results in a marked destruction of furfuraldehyde-yielding materials [Preece, 1940]; the extent of the destruction is such as to make the use of this pretreatment undesirable, even though it may reduce the lignin contamination of subsequent hemicellulose preparations. At the same time, some polyuronide material may pass into the alcoholic extract [see, e.g., Preece, 1931]. The nature of this material has hitherto been obscure, though there has been little reason to suppose that it differed greatly from hemicelluloses prepared by more normal means of extraction. The preparation of the so-called 'lignosaccharide' has now been studied in some detail and, as will appear, it is clear that it does not represent a special type of cell-wall constituent.

Hemicellulose nomenclature has been extremely confused, and it seems that any mention of these substances should be accompanied by a precise definition of what is intended. The position has been reviewed by Norman [1937], who emphasizes that material passing into hemicellulose preparations has a double origin. There is reason to suppose that the 'crude cellulose' remaining after alternate chlorination and sulphite treatment of plant materials represents the essential structural material of the tissues present; it is regarded as comprising 'pure' cellulose with associated cellulosan, the latter in hard-woods appearing to consist very largely of xylan. A variable proportion of this xylan is soluble in dilute NaOH, as is also a proportion of glucosan material. Whether this readily soluble glucosan should be regarded as cellulosan is not clear. Again, the crude cellulose is associated with encrusting materials which are removed by chlorination. These encrusting materials also are soluble to a greater or less degree in dilute alkali and so contribute pentosan, uronic anhydride and possibly in some cases hexosan to the hemicellulose. If this view is correct, therefore, the mixed nature of hemicellulose preparations can hardly be contested, and further evidence that the preparations are mixtures is presented below. It might be suggested that the term hemicellulose should be reserved for the encrusting urono-polysaccharides, the cellulosans being regarded merely as part of the true native cellulose. However, it is perhaps too soon to endeavour to define the various types rigidly and, in general, Norman's tentative nomenclature will be followed, hemicellulose implying urono-polysaccharide material soluble in dilute alkali, irrespective of its origin, though excluding pectin and materials capable of extraction with water.

EXPERIMENTAL

Of the two wood samples used, the mahogany sawdust was from a commercial timber. The willow material used was from two 5-6-year-old branches cut from a growing tree, the tissues down to the wood being removed and discarded before preparation of the sawdust; in this case, a period of some 3 months elapsed between the cutting from the tree and the preparation of the analytical material.

Alcoholic-NaOH extraction

The mahogany material was extracted successively with ether, 95 % ethyl alcohol, 0·5 % ammonium oxalate solution and 95 % alcohol, each extraction being carried out at boiling temperature for 1 hr. and being followed by appropriate washings on a cloth filter. The loss of dry matter was 8·96 %. A modified and probably more efficient pretreatment of the willow sawdust involved successive extractions with 95 % alcohol, 0·5 % ammonium oxalate solution (twice), distilled water and 95 % alcohol (twice), each extraction being for 1 hr. as before; the dry weight loss was 5·77 %. It may be noted that the willow material used all passed a 20-mesh sieve; the mahogany was used as received. The following treatments and determinations were carried out on the air-dried pre-extracted materials:

(*a*) Furfuraldehyde was determined directly on 0·5–0·7 g. of the prepared wood, the distillation being made in presence of 25 ml. each of *N* NaOH and *N* HCl.

(*b*) A similar weight of the wood was extracted with 100 ml. of boiling x % ethyl alcohol-1 % NaOH for 1 hr. The NaOH was neutralized by adding 25 ml. *N* HCl, and the alcohol was removed *in vacuo*. Furfuraldehyde determination was carried out after the necessary adjustment of volume. The values of x used were successively 10, 30, 50, 70 and 90 %.

(*c*) Extractions were carried out as in (*b*), the mixture then being filtered at the pump and the wood residue washed (using small portions of liquor for each wash) with (i) 50 ml. of liquid having the same composition as that used for extraction, and (ii) 75 ml. of aq. alcohol of the same concentration as that already used. The residue was transferred to the distillation flask and 25 ml. each of *N* NaOH and *N* HCl were added, furfuraldehyde distillation then being proceeded with.

(*d*) Owing to absorption of alkali by the wood, the 25 ml. of *N* HCl added in (*b*) did not give a neutral mixture. Since the figures obtained for 'extract' (*b*) – (*c*) were somewhat anomalous with the 70 and 90 % alcoholic treatments, it was suspected that this might be due to a loss of furfuraldehyde during the vacuum distillation, as a result of the somewhat acid conditions prevailing. Accordingly, samples of wood were refluxed with 100 ml. of alcoholic-NaOH as in (*b*), and the cooled mixture was titrated with *N* HCl by the conductometric method. Assuming that the titre required was y ml., addition of HCl was continued to 25 ml.; $(25-y)$ ml. of *N* NaOH were then added, the further treatment being as in (*b*). This treatment was applied only to willow. The end-point was by no means sharp, though a good approximation was probably obtained. The absorption of alkali corresponded to 2·0–2·2 ml. of *N* NaOH per g. of wood.

The results of the treatments, calculated as furfuraldehyde % of the original, dry, unextracted woods, are shown in Table 1. The standard deviation of the mean of quadruplicates is ± 1·06 % of that mean for mahogany, and ± 0·61 % of that mean for willow; the greater precision in the latter case is due to the greater homogeneity of the sample. The standard deviations of differences, (*a*) – (*b*) and (*b*) – (*c*), are calculated independently in each case from the standard deviations of the pair of quadruplicate means concerned.

It will be observed that the results of treatment (*d*) do not differ significantly from those of (*b*), though it may be more than coincidence that degradation

Table 1. *Furfuraldehyde as percentage of original wood*

	Control (*a*)	Residue + extract (*b*)	Residue + extract (*d*)	Residue (*c*)	Degradation loss (*a*) – (*b*)	Extract (*b*) – (*c*)
Mahogany	9·17	—	—	—	—	—
1% NaOH, alcohol 10%	—	8·53	—	7·62	0·64 ± 0·132	0·91 ± 0·121
30%	—	8·70	—	7·99	0·47 ± 0·134	0·71 ± 0·125
50%	—	8·70	—	8·42	0·47 ± 0·134	0·28 ± 0·128
70%	—	8·60	—	8·61	0·57 ± 0·133	—
90%	—	8·56	—	8·74	0·61 ± 0·133	—
Willow	14·55	—	—	—	—	—
1% NaOH, alcohol 10%	—	13·00	13·08	10·38	1·55 ± 0·119	2·62 ± 0·101
30%	—	13·14	13·20	11·32	1·41 ± 0·120	1·82 ± 0·106
50%	—	13·24	13·26	12·28	1·31 ± 0·120	0·92 ± 0·110
70%	—	13·12	13·14	13·39	1·43 ± 0·120	—
90%	—	13·04	13·19	13·08	1·51 ± 0·120	—

with (*d*) is represented by smaller values throughout than with (*b*). However, (*d*) need not be discussed further.

The similarity of behaviour of the two woods is very great. With both the degradations losses show little change with variations in alcohol concentration, though the loss with willow is relatively and absolutely greater than with mahogany. The extract behaviours are likewise similar, though willow yields a considerably greater amount of material soluble in 10, 30 and 50 % alcoholic-1 % NaOH. There is a slight irregularity in the results from the 70 and 90 % extracts, but the figures obtained for the difference (*b*) – (*c*) are not significant (with the possible exception of the 70 % willow extract) and this matter may at present be ignored. The extracts containing up to 50 % of alcohol have removed the equivalents of amounts of furfuraldehyde which diminish as the alcohol concentration increases, and it is apparent that the extraction of furfuraldehyde-yielding material has substantially stopped by the time the alcohol concentration reaches 70 %. The regular decrease in extraction appears to support the contention that we are not dealing here with any special type of hemicellulose.

Working with straws, Norman [1935] reported an apparently greater extraction of polyuronides with 30 % alcohol-1 % NaOH than with aq. 1 % NaOH. With willow, aq. 1 % NaOH gave an extract furfuraldehyde of 2·40 % with a degradation loss of 2·16 %. The degradation loss is significantly greater than in any of the alcohol treatments, and it is obviously extremely difficult to attempt comparison between the present results and those of Norman, which took no account of the destruction of furfuraldehyde. Comparison is rendered further inadvisable by the fact that crude straw was used, whilst the willow had undergone preliminary treatment which included extraction with alcohol. Balancing any repressive effect that alcohol may have on degradation, it has previously been shown [Preece, 1940] that material soluble in alcohol may protect furfuraldehyde-yielding material against destruction; it may well be that such alcohol-soluble material also impedes extraction of hemicelluloses, dissolution being then favoured by even low concentrations of alcohol in the extracting medium.

Qualitative extraction

The following general method of 'lignosaccharide' and hemicellulose preparation was applied to fresh samples of both sawdusts, 750 ml. of each extractant being used per 100 g. of wood. Extraction was carried out at boiling temperature for 1 hr. each with: (i), (ii) aq. ammonium oxalate solution; (iii) distilled water; (iv), (v) x % alcohol-1 % NaOH; (vi), (vii) x % alcohol. The wood residue was dried in air and reserved; it is designated lr_x. The values of x used were 10, 30 and 50 %. Extracts (i), (ii), (iii) and (vii) were discarded, but (iv), (v) and (vi) were combined and filtered through pulp. The volume of N HCl necessary to adjust the whole to neutrality was determined by titration of an aliquot portion by the minimum conductance method. The neutralized extract was concentrated under reduced pressure to about 1/4 vol., further concentration not being attempted since there is reason to suppose that high salt concentrations may interfere with hemicellulose precipitation. An equal volume of acetone was added and the precipitate, l_x, was purified as described later. Precisely one-half of the residue lr_x was extracted for successive 2-hr. periods with (viii), (ix) and (x) aq. 4 % NaOH at room temperature; repeated washings with water were then applied, after which the residue—hr_x—was dried in air and reserved. Hemicelluloses—h_x—were precipitated from the combined and filtered NaOH extracts by acidifying with HCl and adding an equal volume of acetone.

A further hemicellulose preparation, h, was made by extracting the wood by methods (i), (ii) and (iii), the extracts being discarded. The residue was then treated as under (viii), (ix) and (x), the hemicellulose being precipitated from these three combined extracts. The washed and dried wood residue is designated hr.

A measure of purification of the mahogany preparations was obtained by dissolving or suspending them in 2 % NaOH and reprecipitating with acetone after acidifying with HCl. The willow preparations, on the other hand, were all passed through the Cu purification method, after filtration of their NaOH solutions. Analyses of the products are shown in Table 2.

Table 2. *Analysis of hemicelluloses*

		On dry, ash-free hemicellulose			Furfuraldehyde on dry, ash-free, lignin-free material
Preparation		Yield, on dry wood %	Lignin %	Furfuraldehyde %	%
Mahogany	h	2·30	4·33	54·26	56·72
	h_{10}	1·90	1·67	53·33	54·24
	h_{30}	2·11	2·23	55·81	57·08
	h_{50}	2·15	1·45	55·21	56·02
	l_{10}	1·72	21·34	31·48	40·02
	l_{30}	0·70	25·47	27·59	37·02
	l_{50}	0·16	35·38	21·90	33·89
Willow	h	10·64	1·06	57·53	58·15
	h_{10}	5·61	0·79	57·34	57·79
	h_{30}	7·00	0·98	57·82	58·39
	h_{50}	8·92	0·85	56·26	56·74
	l_{10}	3·07	7·22	48·12	51·92
	l_{30}	1·38	6·03	49·41	52·58
	l_{50}	0·22	11·90	37·63	42·71

Much of the lignin of the mahogany preparations of the l_x series has been carried down mechanically; thus, mahogany l_{10} was purified from a filtered NaOH solution by Cu treatment and then gave (on the ash-free basis): furfuraldehyde 38·09 %, lignin 5·13 %. Correcting the furfuraldehyde result for the lignin content the figure becomes 40·15 %; evidently the purification has not modified the composition of the lignin-free material, of which 82 % was recovered by the technique adopted. It is clear that alcohol and acetone washes alone cannot be relied on for removal of lignin. The alcoholic-NaOH pretreatment has markedly reduced the lignin content of mahogany preparations h_x as compared with h; with willow the corresponding differences are insignificant. It appears that, with both woods, the Cu-purified preparations l_x give markedly more lignin than the corresponding members of the h_x series. The variations in furfuraldehyde yield for h and h_x from a given wood are small and somewhat irregular; these furfuraldehyde yields always exceed those for l_x, even after correction for lignin. The yields of l_x show again the restricting effect of increasing alcohol concentration.

The wood residues

The seven residues from each wood, and the original woods themselves, were examined for furfuraldehyde yield and for their content of Cross and Bevan cellulose, the furfuraldehyde yield from this crude cellulose being also determined. No pretreatment was applied in the cellulose determination, except that the original wood samples were extracted with alcohol-benzene mixture. Chlorination was for periods of 20 and 15 min., followed by further treatments as required of 10 min. each. The results are shown in Table 3, where the compositions of the crude celluloses are also given, it being assumed that the furfuraldehyde is derived exclusively from xylan, pure cellulose (glucosan) being obtained by difference.

Table 3. *Analysis of wood residues*

	Wood	lr_{10}	lr_{30}	lr_{50}	hr_{10}	hr_{30}	hr_{50}	hr
Mahogany:								
On dry wood or residue, %:								
Total furfuraldehyde	9·30	9·88	9·95	10·01	8·22	8·19	8·38	8·20
Cross and Bevan furfuraldehyde	5·02	6·84	6·86	6·71	6·23	6·37	5·68	5·92
Chlorination loss	4·28	3·04	3·09	3·30	1·99	1·82	2·70	2·28
Cross and Bevan cellulose	53·64	63·92	62·63	61·34	65·30	67·75	65·01	62·55
On Cross and Bevan cellulose, %:								
Glucosan	85·5	83·4	83·0	83·0	85·2	85·4	86·4	85·3
Para-xylan	14·5	16·6	17·0	17·0	14·8	14·6	13·6	14·7
Willow:								
On dry wood or residue, %:								
Total furfuraldehyde	14·49	14·12	15·30	15·68	8·37	9·40	9·27	8·81
Cross and Bevan furfuraldehyde	9·73	12·43	12·88	13·00	7·54	8·13	7·89	7·17
Chlorination loss	4·76	1·69	2·42	2·68	0·83	1·27	1·38	1·64
Cross and Bevan cellulose	59·93	73·09	72·18	70·58	70·45	71·56	70·00	68·13
On Cross and Bevan cellulose, %:								
Glucosan	74·8	73·6	72·5	71·4	83·4	82·4	82·5	83·7
Para-xylan	25·2	26·4	27·5	28·6	16·6	17·6	17·5	16·3

The figures for Cross and Bevan cellulose on dry wood or residue indicate the relative efficiencies of extraction of cellulosic and non-cellulosic materials. Thus, with both woods, increasing concentration of alcohol in the alcoholic-NaOH pretreatments diminishes the proportionate extraction of non-cellulose, whilst with mahogany the subsequent extraction with 4% NaOH further enhances the cellulose content of the residue, this extractant having here removed proportionately more non-cellulose than cellulose. With willow, on the other hand, the result of 4% NaOH extraction after alcoholic-NaOH pretreatment is to diminish the cellulose content of the residue; clearly, in this case, cellulose has been extracted more effectively than non-cellulose.

Again, no matter what pretreatment or extraction has been applied, chlorination always removes more furfuraldehyde-yielding material from the residue. Two possible explanations suggest themselves; either the various NaOH treatments increase the susceptibility of the para-xylan to the effects of chlorination and sulphite extraction, or these NaOH treatments fail to remove the whole of the encrusting hemicelluloses. Whilst the first alternative is not to be overlooked, there is direct evidence to support the second; thus, Preece [1931] has shown that hot alkaline extraction of wood residues may continue to remove urono-pentosan after exhaustive extraction in the cold has failed to take any further appreciable amount into solution.

The variations found in the compositions of the various Cross and Bevan celluloses are of the first importance, and it may be remarked that a difference in para-xylan content exceeding 0·5 % between two samples may be considered significant. With mahogany, the compositions of the celluloses from the original wood and from the residues hr, hr_{10} and hr_{30} are substantially identical. Clearly this result by itself is most misleading; it must simply be implied that xylan and glucosan have been proportionately removed. It is apparent that, with this wood (and with willow also), hot alcoholic-NaOH removes glucosan more effectively than xylan, whilst with cold 4 % NaOH the reverse is true. Thus, the observed cellulose compositions depend only on the differing rates of extraction of the components; there is every reason to believe that both components are removed to greater or less extents by the various NaOH treatments applied. These results, especially in so far as they relate to hot alkaline extraction, may be compared with those recently obtained by Mackney [1940].

DISCUSSION

The figures of Table 1 amply confirm the destructive effect of boiling alcoholic-NaOH on furfuraldehyde-yielding substances, and it is interesting to note that the magnitude of the loss, while but little affected for a given wood by the alcohol concentration, does vary considerably with different woods. Thus, for 50 % alcohol-1 % NaOH the losses for teak, mahogany and willow are respectively 4·2 % [Preece, 1940], 5·1 % and 9·0 % of the total furfuraldehyde, these being the figures when a pretreatment including alcohol extraction had been given. It appears that there may be a further source of loss. In Table 4 are given the calculated (Tables 1 and 2) and actual (Table 2) yields of hemicellulose soluble in alcoholic-NaOH. The yields obtained fall very short of those calculated and it is probable that depolymerization of some of the hemicellulosic material renders it incapable of precipitation by the usual means. More direct evidence of loss of hemicellulose is provided by noting that direct extraction of willow with 4 % NaOH gave a 10·64 % yield of hemicellulose; in no case with this wood did the combined yields of l_x and h_x approach this figure. On the other hand, the direct yield from mahogany is exceeded by $(l_{10}+h_{10})$ and by $(l_{30}+h_{30})$. There can be no doubt that the alcoholic-NaOH treatments do cause the dissolution of more hemicellulose than would be the case with direct cold extraction alone, but whether this increased dissolution is reflected in the yields obtained depends entirely on the susceptibility to degradation of the material concerned.

Table 4. *Hemicellulose yields*

	From mahogany		From willow	
Preparation	Calc. %	Actual %	Calc. %	Actual %
l_{10}	2·89	1·72	5·44	3·07
l_{30}	2·58	0·70	3·68	1·38
l_{50}	1·27	0·16	2·44	0·22

The evidence is complete that hemicelluloses without exception contain material (cellulosan) which is resistant to chlorination and also material (encrusting hemicelluloses) which is removed by this treatment. Indeed, in the case at least of the willow preparations, the amount of cellulosan present probably predominates. Table 3 indicates that the residues of the lr_x series yield more crude cellulose than the corresponding members of the series hr_x. This can only be the case if cellulosic material is removed more completely than non-cellulose (including encrusting hemicelluloses) by the intervening NaOH extraction. In fact, with the figures given, the cellulosan passing into the hemicellulose extract must exceed twice the amount of non-cellulosan, not all of which need be encrusting hemicellulose. Certainly, however, the differences between willow pairs are not significant except in the case of lr_{10} and hr_{10}; nevertheless, this scarcely affects the above argument. The influence of direct cold extraction with 4 % NaOH on furfuraldehyde-yielding cellulosan materials is shown by a comparison of the para-xylan figures for the cellulose of willow wood and that of the residue *hr* (Table 3).

Hot extraction likewise gives mixtures of hemicelluloses, but the tendency here is towards increased removal of non-pentosan. The relatively low figures for 'lignosaccharide' furfuraldehyde thus appear to be largely accounted for by relatively high glucosan contents, though there may also be differences in uronic anhydride, a point which has not here been investigated. 'Lignosaccharide', therefore, does not differ from hemicelluloses extracted in the cold, except in the proportions of the various components of the mixture. It may be noted that hot extraction, which favours the dissolution of glucosan, also gives fractions showing higher lignin figures when examined by the Schwalbe method; a difference persists even when careful Cu purification is applied. It is possibly worthy of investigation whether these fractions contain high proportions of material either (i) chemically associated with lignin or (ii) of such modified properties as to give products resistant to 72 % H_2SO_4-18 % HCl treatment.

It is abundantly clear therefore that the methods of hemicellulose preparation at present employed give mixed products. It has been claimed on various occasions to separate preparations into fractions that were 'chemically and physically distinct' [Norris & Preece, 1930]; exception has been taken to such claims, but it has never been implied that the fractions were homogeneous. Again, Preece [1931] showed that the analysis of box-wood hemicellulose could be interpreted as indicating a relatively simple composition; but here again there was no suggestion that a simple 'pure' product was, in fact, concerned. The dangers of the problem are further indicated by comparing the results for maize-cob hemicelluloses obtained by Preece [1930] with those for the same materials of Angell & Norris [1936]. The latter authors show that, by careful control of *p*H, Preece's fractions A_1, B_1 and C_1 can be precipitated as a single fraction. However, A_1, B_1 and C_1 show distinct differences in chemical composition, even though the partial separation achieved may have been fortuitous; evidence of the possibility of even a partial fractionation would appear to be more informative than the demonstration that, under other conditions, separation is avoided. Further, in relation to these maize-cob products, it now seems certain that their chemical analysis is to be accounted for on the assumption that the products of hydrolysis are pentose, uronic acid and glucose.

SUMMARY

1. Loss of hemicellulose material as a result of boiling alkaline pretreatment is confirmed.

2. Hot alkaline extraction of woods favours the dissolution of non-pentosan; extraction in the cold favours the dissolution of xylan.

3. Hemicellulose preparations contain materials derived both from the encrusting hemicelluloses and from the cellulose fraction; the relative proportions of the components of the mixture vary with the conditions of extraction and with the raw material.

4. The mixed nature of hemicellulose preparations is stressed.

The author's best thanks are due to his colleagues, Messrs J. W. Ingham and James Mathieson, for valued assistance and advice.

REFERENCES

Angell & Norris (1936). *Biochem. J.* **30**, 2155.
Mackney (1940). *J. Coun. sci. Industr. Res., Aust.*, **13**, 115.
Norman (1935). *Biochem. J.* **29**, 945.
—— (1937). Biochemistry of Cellulose, Polyuronides, Lignin, etc. Oxford.
Norris & Preece (1930). *Biochem. J.* **24**, 59.
Preece (1930). *Biochem. J.* **24**, 973.
—— (1931). *Biochem. J.* **25**, 1304.
—— (1940). *Biochem. J.* **34**, 251.

69. HISTIDINE IN URINE. 2

By E. RACKER

From the Biochemical Laboratory, Cardiff City Mental Hospital

(Received 31 May 1941)

In a previous paper [1940] a method has been described for quantitative estimation of histidine in urine. Numerous estimations have been carried out and values corresponding to 2–80 mg. histidine monohydrochloride per 100 ml. have been found in normal urines. These figures were unexpectedly high, and so it was decided to investigate further the nature of the substance or substances responsible for the bromine colour test.

EXPERIMENTAL

1. *Purification of the substance giving the bromine colour test*

Urine, in portions of 200–300 ml., was shaken with 20 g. purified animal charcoal for 25 min. The urine was then filtered through a Büchner funnel and the filtrate poured into a separate flask. The charcoal, which remained in the Büchner funnel, was washed with $N/10$ H_2SO_4 until all the histidine adsorbed by the charcoal was eluted and the last washing became negative to the colour test. The first urine filtrate, which was kept separate, was again shaken with charcoal, filtered and the filtrate kept separate, the charcoal being washed with $N/10$ H_2SO_4. This procedure was repeated until the original urine was free from histidine. The combined washings were then evaporated *in vacuo* to about 100 ml. and treated with hot saturated baryta until neutral to phenol red. The precipitated barium sulphate was centrifuged down, the supernatant fluid decanted, the precipitate washed with hot distilled water and again centrifuged. The combined supernatant fluids were evaporated *in vacuo* to about 50 ml. and 5 ml. N H_2SO_4 were added. To this an excess of $AgNO_3$, dissolved in distilled water, was added (6–8 g. were usually required). After precipitation was completed, the mixture was filtered, the precipitate of silver chloride being washed with distilled water. The clear filtrate was brought to pH about 8·0 with $2N$ NaOH and left overnight in the refrigerator. The precipitate was filtered off and taken up in distilled water. It was then acidified with dilute H_2SO_4 and decomposed with H_2S for about 12 hr. The mixture was filtered, the precipitate washed and the combined filtrates evaporated *in vacuo* to a low bulk. This purified solution was analysed by the bromine reaction and diluted until it corresponded with a 1 % solution of histidine monohydrochloride. The purified material was used for further investigation.

2. *The biological breakdown of the substance in urine*

It has been shown by Edlbacher [1926] that liver contains an enzyme, histidase, which breaks down histidine. The activities of this enzyme in breaking down histidine and the substance excreted in urine have been compared.

The liver was passed through a Latapie mincer, then ground with sand and suspended in $M/15$ Na phosphate buffer of pH 8·0, so that 4 ml. of the suspension contained 500 mg. of liver brei. The suspension was centrifuged for 20 min., the supernatant fluid decanted and used as enzyme solution. 4 ml. were used for each experiment. The activity of the enzyme was noted, comparing the breakdown of 10 mg. histidine monohydrochloride with that of the corresponding amount of the purified urine solution over varying periods. After incubation in a water bath at 40° the mixture was precipitated with 5 ml. of

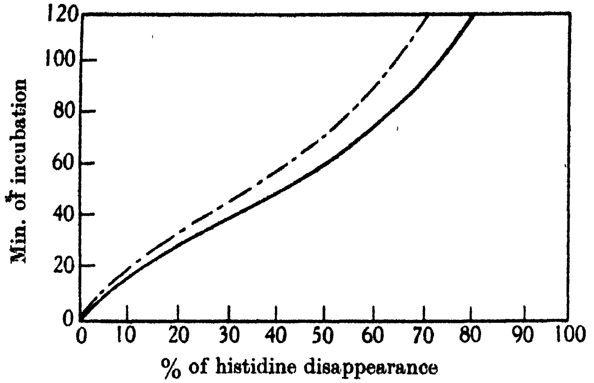


Fig. 1. Rat liver histidase activity. —— Histidine HCl. —·— Urine histidine.

20 % trichloroacetic acid and centrifuged; the supernatant fluids were decanted and the precipitate twice washed with distilled water. The fluids were collected and made up to a known volume. The clear solutions were analysed by the bromine reaction and the results obtained are shown graphically in Fig. 1.

3. *Pharmacological test of the substance in urine*

Hanke & Koessler [1922; 1924] studied the formation of histamine from histidine by various types of *Bact. coli*. Recently Gale [1940] has followed this breakdown under more favourable conditions in manometric experiments. Using suspensions of 'resting bacteria' [Quastel & Whetham, 1924] he measured the activity of the decarboxylase for various amino-acids.

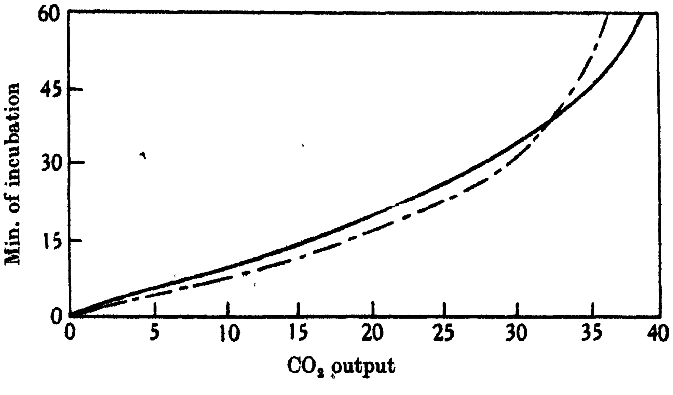


Fig. 2. Decarboxylation of histidine. —— Histidine HCl. —·— Urine histidine.

The decarboxylation of the substance in urine has been followed by this method, using a standard histidine solution as control. After the manometric experiments were concluded the solutions were tested pharmacologically.

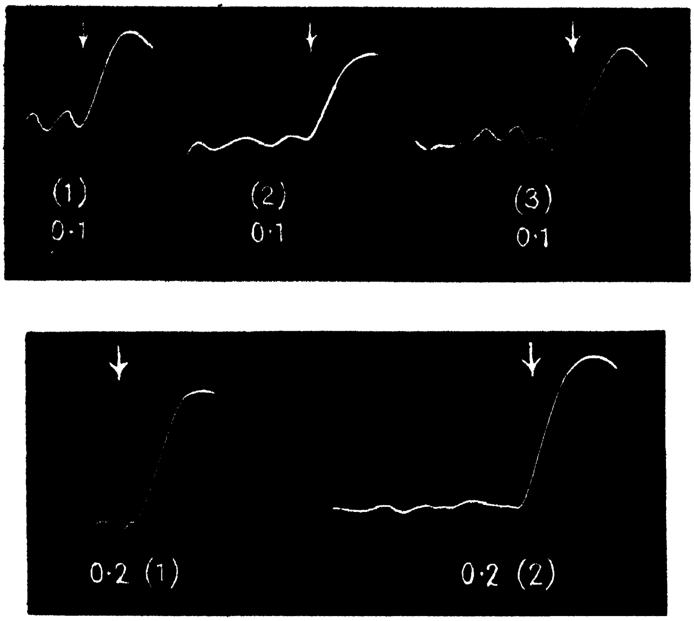


Fig. 3. Fowl caecum. (1) 0·1 μg. histamine HCl. (2) 0·1 μg. histamine from histidine HCl. (3) 0·1 μg. histamine from urine histidine. Lower figure: 0·2 μg. histamine HCl.

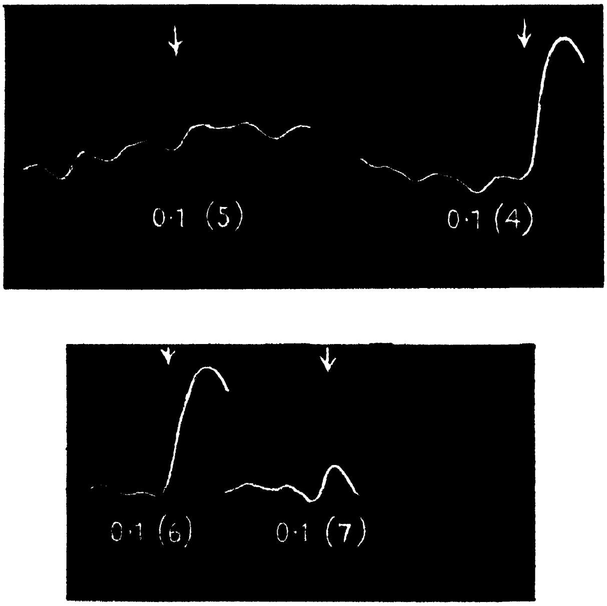


Fig. 4. Fowl caecum. (4) 0·1 μg. histamine from urine histidine. (5) 0·1 μg. histamine from urine histidine + histaminase. (6) 0·1 μg. histamine HCl. (7) 0·1 μg. histamine HCl + histaminase. All incubated for 24 hr. at 37° and then tested.

The respiratory experiments have been carried out under the conditions laid down by Gale. The bacteria (*Bact. coli Esch.* Nat. Inst. of Type Cultures, no. 86) were grown in 2 % glucose broth at 27° for 18 hr., centrifuged, well washed and suspended in distilled water. The dry weight was determined in each case by a photoelectric turbidimeter. 1 ml. of the bacteria suspension was pipetted into each Warburg vessel with 1 ml. of phthalate buffer (*p*H 4·0) and 0·7 ml. distilled water. 0·3 ml. of the histidine or urine solution was placed in the side cup. After equilibration in the thermostat at 30° the histidine was tipped in. The results were recorded graphically (Fig. 2).

After incubation the bacteria were centrifuged off, the clear solutions neutralized and diluted with Tyrode solution to a concentration of histamine 1 μg./ml. according to the theoretical amounts of histamine formation to be expected from the CO_2 output.

The solutions were then tested on the isolated caecum of the fowl [Barsoum & Gaddum, 1935]. The gut was suspended in a small water bath containing 8 ml. of a Tyrode glucose solution, kept at 37° and continuously aerated with oxygen. The amount of fluid tested did not exceed 0·2 ml. The contractions of the gut were recorded on a slowly rotating smoked drum and typical results are given in Fig. 3.

Histaminase (Torantil Bayer), which breaks down histamine, acts in the same way on the histamine produced from normal urine (Fig. 4).

4. *Estimation of histidine by nitranilic acid*

Block [1940] has shown that histidine is quantitatively recovered from solutions as nitranilate and he has used this for estimation of the amino-acid. This method was applied to the purified urine solution and quantitative recoveries, corresponding to the bromine colour test, have been obtained. The experimental procedure was as follows.

The purified urine solution was first treated with an excess of 95 % alcohol and centrifuged. The precipitate was dissolved in distilled water, reprecipitated with alcohol and centrifuged. This process was repeated until the precipitate was free of histidine. The collected supernatant fluids were evaporated *in vacuo* to a few ml. and an equal amount of absolute methyl alcohol was added. To this the nitranilic acid, dissolved in distilled water, was added in excess. The mixture was left in the refrigerator overnight, and the centrifuged precipitate was then dried to constant weight. The result obtained is given in Table 1.

Table 1

	Histidine present mg.	Dry weight of precipitate mg.	Recovery %
Histidine HCl	40	71·8	97·5
Urine histidine	40	76·8	104·2

DISCUSSION

The experimental evidence presented above indicates that the greater part of the substance in urine reacting with bromine is histidine. Recent work on the subject [Sakaguchi, 1940; Langley, 1941] is in agreement with this conclusion.

Tschopp & Tschopp [1938] have investigated the excretion of histidine in various diseases, but as they were working with a qualitative method the results appear not to be conclusive.

A small number of quantitative histidine estimations have been made on patients with malignant tumours, relating the histidine excretion to the specific gravity and urea excretion; so far normal figures have always been obtained.

Two pathological conditions are of special interest with regard to histidine metabolism.

1. Experimental shock, produced by a procedure described elsewhere [Quastel & Racker, 1941]. Measurements of histamine formation *in vivo*, and by tissues anaerobically *in vitro*, have not, however, been convincing enough to justify any conclusion.

2. *Bact. coli* infection. Considering the large amounts of histidine excreted normally and the relatively small amount of histamine necessary to exert a pharmacological effect, investigation of the fate of urinary histidine seems to be indicated. The acidity of the urine prevailing in *Bact. coli* infection of the urinary tract should be a favourable factor for histamine formation. Because of lack of suitable clinical material this investigation has not yet been carried out.

It may be noted that in severe cases of toxaemia of pregnancy histamine has been found and isolated from urine by Kapeller-Adler [1941], thus indicating an altered histidine metabolism in this condition.

SUMMARY

1. The procedure of purification of the substance in urine, giving rise to the bromine colour test, is described.

2. The substance is broken down by liver histidase.

3. The decarboxylation of the substance has been followed in manometric experiments and the fluids have then been tested for their action on the isolated fowl's gut. If incubated with histaminase the effect on the gut is nullified.

4. The nitranilic acid precipitation method has been applied to the urine substance.

5. It is concluded that the colour produced by the bromine reaction is due to histidine. The clinical significance of this is discussed.

The author wishes to express his indebtedness to Dr J. H. Quastel for his interest and advice throughout this work.

REFERENCES

Barsoum & Gaddum (1935). *J. Physiol.* **85**, 1.
Block (1940). *J. biol. Chem.* **133**, 67.
Edlbacher (1926). *Hoppe-Seyl. Z.* **157**, 106.
Gale (1940). *Biochem. J.* **34**, 392.
Hanke & Koessler (1922). *J. biol. Chem.* **50**, 131.
—— —— (1924). *J. biol. Chem.* **59**, 835, 855.
Kapeller-Adler (1941). *Biochem. J.* **35**, 213.
Langley (1941). *J. biol. Chem.* **137**, 255.
Quastel & Whetham (1924). *Biochem. J.* **18**, 519.
—— & Racker (1941). *Brit. J. exp. Path.* **22**, 15.
Racker (1940). *Biochem. J.* **34**, 89.
Sakaguchi (1940). *J. Biochem., Tokyo*, **31**, 289.
Tschopp & Tschopp (1938). *Biochem. Z.* **298**, 206.

70. A METHOD FOR THE MICRO-ESTIMATION OF IRON IN BIOLOGICAL MATERIALS

By R. H. THORP

From the Wellcome Physiological Research Laboratories, Beckenham, Kent

(*Received 23 April 1941*)

A METHOD for the estimation of very small quantities of iron in biological materials has been in use in this laboratory for some years. It is based upon the reaction of ferrous Fe with $\alpha\alpha'$-dipyridyl described by Hill [1930]. The method has proved so useful for the study of Fe in biological materials of all kinds, such as bacterial cultures, media and tissues of animal origin, that it seems worthy of wider use. When the concentration of the red dye produced by the reaction of ferrous Fe and $\alpha\alpha'$-dipyridyl is measured colorimetrically it is possible to estimate 0·2 μg. Fe per ml. of solution under examination. The sensitivity of the method is approximately the same as that of the thiocyanate method for the estimation of ferric Fe. The presence of Cu, Zn and other metals does not interfere with the test when the quantities of these elements are comparable with that of the Fe [Hill, 1930]. Cu gives a blue colour, but this does not interfere with the estimation of Fe, even when present in amounts up to 1 mg./ml., as a green light filter is used in the colorimeter and the green light is not absorbed by the blue colour due to the Cu. Zn forms a colourless compound with $\alpha\alpha'$-dipyridyl, but this does not affect the Fe reaction when the reagent is present in excess. Although by theory three molecules of $\alpha\alpha'$-dipyridyl combine with one atom of Fe (in molar equivalents, 468 g. of $\alpha\alpha'$-dipyridyl to 56 g. of Fe), in practice it has been found best to use ten times more dipyridyl than the theoretical amount. Since the red complex produced in the presence of excess $\alpha\alpha'$-dipyridyl is dissociated over a range of *p*H 3·5–8·5, it is satisfactory to estimate the colour at about *p*H 6. In our method the standard solutions have a *p*H of 6·3. Hill has shown that Fe may be estimated in many biological materials without previous ashing if precautions are taken to prevent the adsorption of the red iron dipyridyl upon insoluble proteins. We have preferred always to ash the materials by the usual wet ashing process, using perchloric acid, HNO_3, or H_2O_2, instead of selenium or catalytic copper methods, for oxidation. The red colour of the complex ion is formed only in the presence of ferrous Fe so that reduction is necessary after ashing the material. This is effected by sulphurous acid formed from sodium sulphite in the presence of HCl. At present $\alpha\alpha'$-dipyridyl is not commercially available but it may be prepared in several ways. Blau [1888] prepared it first, from copper picolinate, and Hill describes a method based upon this reaction. Hein & Retter [1928] devised a simpler method of preparation by the oxidation of pyridine in a closed tube. We have found it convenient to use the method of Wibaut & Overhoff [1928], in which 2-bromopyridine is boiled with cymene in the presence of copper powder for 1½ hr. The cymene is then distilled off in steam, the residue made alkaline and finally the $\alpha\alpha'$-dipyridyl is distilled. The product is purified by recrystallizing from 20 % alcohol.

METHOD

Reagents

Iron-free HCl. 20 % HCl is distilled in an apparatus made entirely of pyrex glass. Two strengths of the acid are prepared from the distillate, *N* and *N*/10; these are standardized and should be within 1 % of the nominal strengths.

αα′-Dipyridyl reagent. An accurately weighed amount of the material is dissolved in sufficient 0·1*N* HCl to make a 1 % solution. This solution is quite stable but should be stored in a white or pyrex glass bottle which has been filled with conc. HCl and allowed to stand for some days, and then repeatedly rinsed in glass-distilled water. Under these conditions the reagent should show no perceptible pink colour.

Sodium sulphite solution. A 20 % solution is prepared freshly as required from A.R. sodium sulphite crystals, $Na_2SO_3, 7H_2O$.

Standard iron solution. A stable stock solution of Fe is prepared from ferric ammonium sulphate by dissolving 0·4318 g. in a little water, adding 100 ml. of *N* iron-free HCl and making up to 1 l. with glass-distilled water. This solution contains 50 μg. Fe/ml.

Standard colours

Standard colours are prepared containing from 0·2 to 10 μg. Fe/ml. The standard solution when diluted five times contains 10 μg. Fe/ml. Appropriate dilutions are made from this by using a micrometer syringe or burette and the volumes adjusted to 1 ml. with distilled water. It is convenient to dilute the solutions in $3 \times \frac{3}{8}$ in. tubes calibrated at 1 ml. To each tube is now added 0·4 ml. of the dipyridyl reagent followed by 0·5 ml. of 20 % sodium sulphite solution and 0·1 ml. of *N* HCl. The red colour reaches a maximum in 15 min. and is permanent; it may be compared optically or photoelectrically. The peak of the

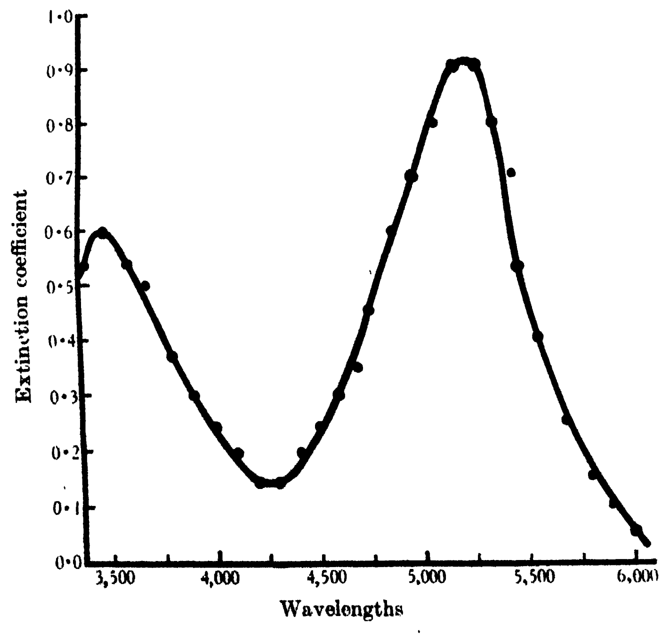


Fig. 1. Spectrographic absorption curve of iron dipyridyl solution.

absorption of the red dye occurs at a wave-length of 5250 Å. In Fig. 1 is reproduced an absorption curve of the red colour of iron dipyridyl, which was kindly furnished by Dr Foster of the Wellcome Chemical Works, Dartford. If a powerful light source is available a Wratten green filter no. 62 or Ilford 'Mercury Green' type of filter is most suitable, but if this is too dense a filter no. 61 is almost as efficient and transmits far more light. The usual convention of plotting the concentration against the logarithm of the percentage transmission is followed. A typical calibration is shown in Fig. 2, derived by using a photoelectric colorimeter employing a gas-filled caesium cell. If the filtered light is truly monochromatic, or nearly so, and is complementary in colour to the absorption band of the solution, this curve will be linear. This is rarely the case unless the filter used is very dense or the solutions being compared are pale in colour. The calibration curve as shown in the figure is, however, quite satisfactory in practice. If the range of standard solutions of Fe used in the calibration extends only to 5 μg./ml. the curve is virtually linear when plotted in the same way. Using a colorimeter employing a selenium cell the sensitivity is lower but a suitable calibration curve can be prepared in the same way over a wide range of Fe concentration. Although this curve is less reliable at lower values of Fe concentration it is equally practicable, and is particularly useful when the range of the concentrations of Fe is large.

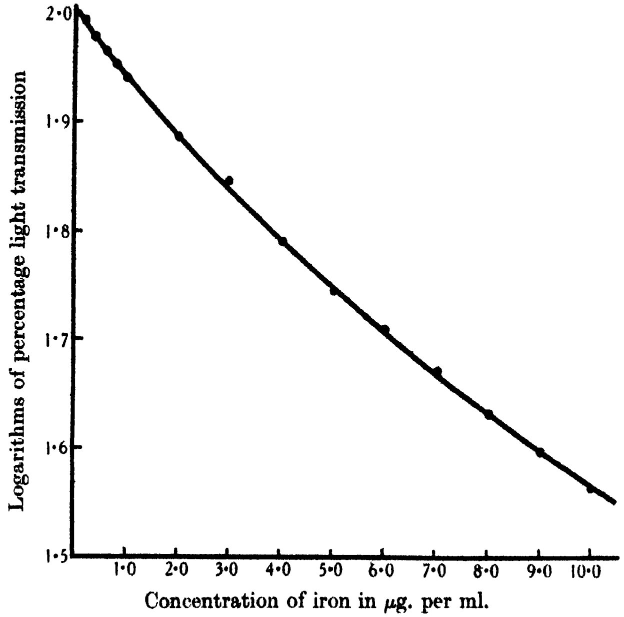


Fig. 2. Calibration curve for photoelectric colorimeter.

Estimation of Fe in samples

For tissues or foodstuffs we have found it convenient to weigh out accurately an amount of material estimated to contain between 10 and 200 μg. Fe and ash by the wet ashing method described by Eden & Green [1940] using H_2SO_4 and perchloric acid. With about 1 g. or less of tissue it is unnecessary to use HNO_3 as an additional oxidizing agent. The excess acid is neutralized with solid Na_2CO_3 (A.R.) until there is no further effervescence. The neutral solution is then reacidified with HCl to about pH 4; the exact value is not vital, owing to the buffering effect of the sulphite in the subsequent stages of the estimation. The solution is then diluted to 50 ml. in a standard flask with glass-distilled water. For the preparation of the coloured solution 1 ml. of the liquid containing Fe is added to 0·4 ml. of the dipyridyl reagent in one of the small tubes and this is followed by 0·5 ml. of sodium sulphite solution and 0·1 ml. of N HCl, as in the case of the standard colours. The mixture is then shaken and the red colour developed is read upon the colorimeter after an interval of 15 min. The concentration of Fe in the solution is then calculated from the calibration curve. In view of the sensitivity of the method it is necessary to prepare control tubes covering the chemicals used in the entire process. The value obtained for the blank is then subtracted from that obtained in the estimations. If the red colour developed is too deep for the range of the colorimeter the solution may be diluted with water until a convenient reading is obtained. Where the Fe content is known to be high by previous estimations it is more convenient to dilute the solution obtained after ashing and neutralization.

SUMMARY

1. A method of estimating small amounts of Fe in biological materials is described. The method is based upon the red complex formed when αα′-dipyridyl reacts with ferrous Fe.

2. Amounts of Fe as small as 0·2 μg./ml. can be estimated colorimetrically or optically.

3. An outline is given of methods for preparing αα′-dipyridyl, since this is not available commercially.

I wish to take this opportunity of thanking Mr R. Sellar for the technical assistance he has given in the chemical work associated with the method.

REFERENCES

Blau (1888). *Ber. dtsch. chem. Ges.* **21**, 1077.
Eden & Green (1940). *Biochem. J.* **34**, 1203.
Hein & Retter (1928). *Ber. dtsch. chem. Ges.* **61**, 1790.
Hill (1930). *Proc. roy. Soc.* B, **107**, 205.
Wibaut & Overhoff (1928). *Rec. Trav. chim. Pays-Bas*, **47**, 761.

71. BIOLOGICAL SYNTHESIS OF OXALOACETIC ACID FROM PYRUVIC ACID AND CARBON DIOXIDE

2. THE MECHANISM OF CARBON DIOXIDE FIXATION IN PROPIONIC ACID BACTERIA[1]

By H. A. KREBS AND L. V. EGGLESTON

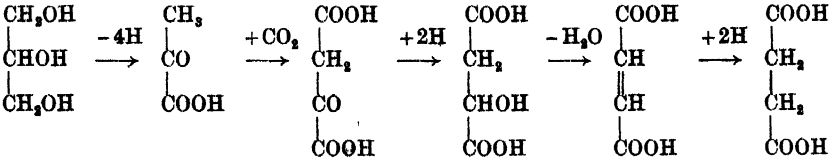
From the Department of Biochemistry, University of Sheffield

(*Received 4 April 1941*)

Wood & Werkman [1936, 1; 1938] found that propionic acid bacteria utilize CO_2 when glycerol is present and that the CO_2 fixation is coupled with the formation of an equivalent amount of succinic acid. These observations are summarized by equation (1):

$$CH_2OH.CHOH.CH_2OH+CO_2=COOH.CH_2.CH_2.COOH+H_2O. \qquad (1)$$

Recently, Wood & Werkman [1940] suggested the following mechanisms for this reaction:



$$\begin{array}{l}CH_2OH\\|\\CHOH\\|\\CH_2OH\end{array}\xrightarrow{-4H}\begin{array}{l}CH_3\\|\\CO\\|\\COOH\end{array}\xrightarrow{+CO_2}\begin{array}{l}COOH\\|\\CH_2\\|\\CO\\|\\COOH\end{array}\xrightarrow{+2H}\begin{array}{l}COOH\\|\\CH_2\\|\\CHOH\\|\\COOH\end{array}\xrightarrow{-H_2O}\begin{array}{l}COOH\\|\\CH\\\|\\CH\\|\\COOH\end{array}\xrightarrow{+2H}\begin{array}{l}COOH\\|\\CH_2\\|\\CH_2\\|\\COOH\end{array}\qquad (2)$$

This scheme is supported by several experimental observations. Wood *et al.* [1937] found that glycerol in accordance with (2) can be converted into pyruvic acid. Wood *et al.* [1940] showed, with the help of the isotope C^{13}, that the fixed CO_2 appears in fact in the carboxyl groups of succinic acid. The experiments reported in this paper were mainly undertaken to supply further evidence in support of scheme (2).

The organism

A culture of *Bacterium acidi propioni* obtained from the National Collection of Type Cultures (No. 4759) showed a very feeble fermenting capacity. The organism[2] grew vigorously under aerobic conditions (on the surface of agar), but it failed to grow anaerobically on glucose-lactate-yeast water, or on whey. Suspensions of washed bacteria, grown on agar, did not attack glucose, lactate or glycerol under anaerobic conditions; pyruvate was slowly decomposed. Attempts to recover the original fermenting power by transferring the organism to liquid media (glucose-lactate-yeast water, whey) were unsuccessful. Even after 2 months no growth or fermentation was perceptible. The Curator of the Collection informed us that the organism had been cultivated for 5 years on agar slopes. This may account for the inability to ferment, for Jensen [1931;

---

[1] A preliminary report appeared in *Chem. and Ind.* **59**, 849 (1940).

[2] This is the organism recently used by Quastel & Webley [1939; 1941]. We can confirm the effects of vitamin B_1 described by Quastel & Webley with this atypical bacterium, but we obtained no vitamin effects with the six strains of propionic acid bacterium used in the present paper.

p. 47] states 'when propionic acid bacteria have become accustomed to aerobic conditions, their fermentative powers are gradually weakened'.

The organism used in the greater part of this work was isolated from Gruyère cheese obtained at a local shop. Material from the 'eye' of the cheese was spread over an agar plate of lactate-yeast water and incubated at 25° in an anaerobic jar filled with CO_2. Among several organisms a typical propionic acid bacterium was obtained. Its properties are as follows:

(1) Very short, non-motile, gram-positive rods, non-spore forming, assuming involution forms in old cultures, or when grown aerobically at 37°. Agar stab culture: abundant stab growth, moderate surface growth.

(2) Lactate is rapidly fermented under anaerobic conditions, yielding 25–30 % CO_2 and 90–100 % fixed acids. Among these propionic acid was identified as mercurous salt [Musicant & Kaszuba, 1939].

(3) Glycerol is slowly fermented with the fixation of CO_2 and formation of succinate, as described by Wood & Werkman.

(4) Acid is produced from glucose, lactose, galactose, mannose, fructose, arabinose.

(5) Neither acid nor gas is produced from starch, sucrose, xylose.

(6) A powerful catalase is present.

The properties listed under (1), (2) and (3) characterize the organism as a *Propionibacterium*. The other properties are identical with those of the species *Propionibacterium Shermanii* [Sherman, 1920; van Niel, 1928; Werkman & Brown, 1933].

During the later stages of this work we received five strains of propionic acid bacteria of the American Collection of Type Cultures through the courtesy of Dr J. G. Davis, National Institute for Research in Dairying, University of Reading. They were *P. Freudenreichii*, *P. Shermanii*, *P. Jensenii*, *P. Thönii* and *P. rubrum*. Some experiments were repeated with these organisms.

Most of the experiments reported in this paper were carried out with suspensions of washed cells. The medium used for the large scale preparation of bacterial suspensions contained the following ingredients:

200 ml. yeast water (200 g. dried brewer's yeast and 800 ml. tap water) heated in the autoclave (120°, 30 min.) and filtered. The liquid contained about 3·5 % dry matter.

200 ml. phosphate buffer (0·1 *M*, *p*H 7·4).

25 ml. tryptic casein digest [Gladstone & Fildes, 1940].

40 ml. 50 % sodium lactate.

500 ml. tap water.

25 g. agar.

Sterile medium was poured into large sterile petri dishes (15 cm. diameter) and these were inoculated with 3–4 ml. of a liquid culture (the above medium without agar). The petri dishes were placed in a vacuum jar. The air pressure in the jar was reduced on the water pump to 15–20 mm. Hg and 400–500 mm. CO_2 were then admitted. The temperature of the incubator was 34°. After 4–5 days the cells were washed off the agar with distilled water, centrifuged, resuspended in water, centrifuged again and finally suspended in a small volume of water. This 'stock suspension' was kept in the refrigerator. In the case of *P. Shermanii* the yield was about 200–250 mg. dry cells per petri dish. For metabolic experiments the stock suspension was diluted with solutions of $NaHCO_3$ or phosphate. The concentrations used are stated in the tables.

The manometric and analytical methods were the same as those described in previous papers from this laboratory. All experiments were carried out at 30°.

d(+)-malate was not metabolized. This is worthy of note since the stereoisomerides of lactate were both fermented at the same rate.

Rate of succinate formation from various substrates

If pyruvate, oxaloacetate and fumarate are intermediates in the formation of succinate from glycerol, as suggested by scheme (2), the succinate formation from these substrates should be at least as rapid as the succinate formation from glycerol. Table 4 shows that such is the case. Although the yield of succinate is much lower when lactate and pyruvate are the substrates, the *rate* of succinate formation from these substances is of the same order as that from glycerol.

Table 4. *Rate of succinate formation from various substrates*

39 mg. bacteria in 4 ml. 0·03 *M* $NaHCO_3$. Initial substrate concentration 0·02 *M*; gas phase CO_2; period of incubation (1 hr.).

Substrate	Glycerol	*dl*-Lactate	Pyruvate	Oxaloacetate	Fumarate or *l*(−)-malate
μl. CO_2 and acid	96	676	532	352	131
μl. succinate	73	95	83	177	372

Fermentation of glycerol

(1) *Formation of fumarate.* In the case of *Bact. coli* it has been possible to demonstrate a simultaneous formation of succinate and fumarate from pyruvate [Krebs & Eggleston, 1940]. In experiments with *P. Shermanii*, using glycerol as a substrate, we obtained similar results when an excess of glycerol was present. Details of the experimental conditions are given in Table 5. The yields of fumarate are rather low, presumably because of the rapid reduction of fumarate in the presence of glycerol (see the following paragraph). The simultaneous formation of fumarate and succinate can be taken as evidence in support of scheme (2) [see Krebs & Eggleston, 1940].

Table 5. *Formation of fumarate from glycerol in* P. Shermanii

64 mg. bacteria in 6 ml. 0·04 *M* $NaHCO_3$: 5 % $CO_2 + N_2$; 2 % glycerol; 21 hr.

	Exp. I	Exp. II
μl. CO_2 formed	1540	1380
μl. succinate formed	586	690
μl. fumarate formed	64	62

(2) *Effects of fumarate and oxaloacetate on the fermentation of glycerol.* When fumarate and glycerol are added together to washed suspensions of *P. Shermanii* the rate of acid and CO_2 production is 2–3 times greater than expected from experiments in which the substrates are added alone (Table 6). The rate of

Table 6. *Effect of glycerol and fumarate on the acid and CO_2 production by* Propionibacterium Shermanii

36 mg. bacteria in 4 ml. 0·04 *M* $NaHCO_3$; CO_2.

Substrate added (final conc.) ...	None	Fumarate (0·02 *M*)	Glycerol (0·02 *M*)	Fumarate (0·02 *M*), glycerol (0·02 *M*)
μl. CO_2 evolved after 20 min.	13	47	30	87
40 min.	30	93	65	212
80 min.	39	142	98	440
200 min.	49	228	236	1385
280 min.	83	444	314	1980
μl. succinate formed (280 min.)	—	1556	346	2008

succinate formation is also increased and the data suggest that the following reaction occurs in the presence of glycerol and fumarate:

glycerol + fumarate → oxidation products of glycerol + succinate.

This scheme, however, does not account for all the observations. When small quantities of fumarate are added the increased CO_2 output and succinate formation is equivalent to a multiple of the fumarate added (Table 7). Thus 112 μl. fumarate cause an increase in CO_2 output of 388 μl. and in succinate formation of 340 μl. The effect of fumarate, therefore, is catalytic. From what is known about the metabolism of fumarate in other cells it is very probable that the catalytic effect is associated with hydrogen transport; it might be due either to the system succinate $\rightleftharpoons$ fumarate, or the system fumarate $\rightleftharpoons$ oxaloacetate. The first possibility, however, can be excluded, for if succinate were reversibly dehydrogenated it should be possible to replace fumarate by succinate, and this is not the case. Addition of succinate has no effect on the rate of acid production from glycerol and the added succinate is quantitatively recovered from the solution (Table 10). These results show that the system succinate $\rightleftharpoons$ fumarate is not a hydrogen carrier under *anaerobic* conditions. Apparently O_2 cannot be replaced by other physiological oxidizing agents. On the other hand oxaloacetate can replace fumarate (Table 7) and the catalytic effect with this substance is even slightly greater than with fumarate. This supports the view that the system oxaloacetate $\rightleftharpoons$ fumarate acts catalytically. Experiments with radioactive carbon [Carson & Ruben, 1940] lead to the same conclusion (see below).

Table 7. *Effect of oxaloacetate and fumarate on the succinate formation in the presence of glycerol*

26 mg. bacteria in 4 ml. 0·04 *M* $NaHCO_3$; CO_2.

Substrate added (final conc.)	... ... 0·1 *M* glycerol	0·1 *M* glycerol, 0·00125 *M* oxaloacetate (=112 μl.)	0·1 *M* glycerol, 0·00125 *M* fumarate (=112 μl.)
μl. CO_2 formed after 100 min.	106	322	280
280 min.	302	730	562
22 hr.	1100	1756	1488
μl. succinate formed	374	814	714
Extra succinate (due to addition of oxaloacetate or fumarate), μl.	—	440	340

(3) *Adaptation to glycerol.* Figures given in Tables 6 and 7 show that the fermentation of glycerol is relatively slow in washed cells grown on a lactate-containing medium. When the lactate of the culture medium is replaced by glycerol, cells are obtained which ferment glycerol much more rapidly (Table 8). The enzyme systems attacking glycerol are thus partly adaptive.

Table 8. *Adaptation to glycerol*

30 mg. washed cells in 3 ml. 0·05 *M* $NaHCO_3$; gas phase, CO_2.

Culture medium	Substrate added	μl. CO_2 formed 4 hr.	20 hr.	μl. succinate formed 20 hr.
Lactate yeast-water	—	41	177	102
,,	0·1 *M* glycerol	115	694	150
Glycerol yeast-water	—	217	631	248
,,	0·1 *M* glycerol	735	1870	732

Effects of oxaloacetate on the fermentation of other substrates

Effects of oxaloacetate similar to that on the fermentation of glycerol were found when mannitol, inositol, arabinose or erythritol were the substrates. The rate of acid formation from these substrates was increased by the addition of oxaloacetate (Table 9). No effects were observed when glucose, lactate or pyruvate were the substrates.

Table 9. *Effect of oxaloacetate on the fermentation of various substrates in* P. Shermanii (*strain of the American Collection of Type Cultures*)

30 mg. cells in 3 ml. 0·05 *M* $NaHCO_3$; CO_2; 5 hr.

Substrates added (final conc.)	µl. acid and CO_2 formed	Extra acid and CO_2 formed from added polyhydric alcohol	
		Without oxaloacetate	With oxaloacetate
None	171	—	—
0·00625 *M* oxaloacetate	375	—	—
Mannitol	291	120	275
Mannitol; 0·0065 *M* oxaloacetate	650		
Inositol	357	186	471
Inositol; 0·00625 *M* oxaloacetate	846		
Arabinose	274	103	171
Arabinose; 0·00625 *M* oxaloacetate	546		
Erythritol	191	20	71
Erythritol; 0·00625 *M* oxaloacetate	446		

Anaerobic metabolism of succinate

Previous investigators [Wood *et al.* 1937] have discussed the formation of propionic acid by decarboxylation of succinic acid and it has been stated that succinate disappears [Wood *et al.* 1937; Fromageot & Bost, 1938] when added together with glucose to suspensions of *P. pentosaceum*. In *P. Shermanii* succinate is not metabolized under anaerobic conditions, either when present alone, or when added together with glucose, glycerol or pyruvate (Table 10). (Aerobically, however, succinate can undergo oxidation.)

Table 10. *Anaerobic metabolism of succinate in* Propionibacterium Shermanii

3 ml. suspension; 27 mg. bacteria; 0·07 *M* $NaHCO_3$; gas phase, CO_2; 370 min.

Substrates added	µl. CO_2 found	µl. succinate found
0·4 ml. 0·1 *M* succinate (=896 µl.)	71	882
0·4 ml. 0·1 *M* glucose	1462	26
0·4 ml. 0·1 *M* glucose; 0·4 ml. 0·1 *M* succinate	1448	942
0·4 ml. *M* glycerol	133	96
0·4 ml. *M* glycerol; 0·2 ml. 0·1 *M* succinate (=448 µl.)	139	536

Alternative mechanisms of succinate synthesis

A second mechanism of succinate synthesis has been suggested by Wood & Werkman [1938; 1940] who assume that a dehydrogenation and condensation of 2 mol. of acetate might lead to succinate:

$$2CH_3.COOH \xrightarrow{-2H} COOH.CH_2.CH_2.COOH. \qquad (3)$$

They arrived at this view from the following considerations. The conversion of pyruvate into acetate and CO_2 is the only defined reaction so far known to yield

CO_2 in propionic acid bacteria under anaerobic conditions. If this reaction were the only source of CO_2, acetate and CO_2 should appear in equimolecular quantities. In fact less acetate than CO_2 is formed under certain conditions [Wood & Werkman, 1936, 2]. To explain this Wood & Werkman assumed that some acetate disappears through reaction (3).

This hypothesis is not supported by direct evidence and we do not regard it as satisfactory. In a number of Wood & Werkman's experiments [1936, 2, p. 619, Table II, exps. 1 and 2] the amounts of succinate formed do not make up the deficit in acetate production, even if it is assumed that all the succinate was formed through reaction (3). Thus the hypothesis does not account for the low yield of acetate. However, we accept Wood & Werkman's idea of a further oxidation of acetate, whilst differing from their views on the course of this oxidation. The data on CO_2 production published by Wood & Werkman and by van Niel [1928] suggest that acetate is oxidized to form CO_2. Since succinate cannot be oxidized under anaerobic conditions, it cannot be an intermediate in the oxidation of acetate. The mechanism of acetate oxidation is still completely obscure.

The ability of acetate to undergo oxidation has already been demonstrated by Stone *et al.* [1936] (see also Table 3). The formation of CO_2 from acetate under aerobic conditions is shown in Table 11. Anaerobically addition of acetate causes an increase of the yield of succinate from oxaloacetate (Table 12). We take this as an indication of a reduction of oxaloacetate at the expense of an oxidation of acetate. The hypothesis of the oxidation of acetate also explains the high values for the ratio propionate/acetate which have often been found. According to the original concept of propionic acid fermentation [Fitz, 1878] 3 triose equivalents are assumed to form 2 mol. of propionic acid and 1 mol. of acetic acid and of CO_2. These quantities have been found in many experiments but there are also many cases where the yield of acetate is low. Wood & Werkman [1936, 2] found the ratio propionate/acetate to be between 2·13 and 14·72. These deviations from the expected value 2 are explained by the assumption that acetate undergoes decomposition to CO_2. The reduction equivalent for this oxidation may be the formation of either propionate or of succinate.

Table 11. *Oxidation and CO_2 formation in the presence of acetate*

P. Shermanii; 10 mg. cells in 3 ml. 0·01 *M* phosphate buffer, *p*H 7·1. Method of Warburg & Yabusoe [1924]; period of incubation 105 min.

Substrate	—	0·03 *M* acetate
µl. O_2 used	135	250
µl. CO_2 (including HCO_3') formed	151	289

Table 12. *Effect of acetate on the fermentation of oxaloacetate*

30 mg. cells, *P. Shermanii*, in 3 ml. 0·04 *M* $NaHCO_3$; 5 % CO_2 in N_2; period of incubation 21 hr. The bacteria were shaken before the experiment for 18 hr. in the presence of O_2 to remove preformed intracellular substrates.

Added substrates	0·5 ml. 0·1 *M* oxaloacetate	0·5 ml. 0·1 *M* oxaloacetate, 0·5 ml. 0·5 *M* acetate
µl. succinate formed	352	446

Experiments with other strains of propionic acid bacteria

Further experiments were undertaken to see whether the reduction of oxaloacetate and the catalytic effects of oxaloacetate on the fermentation of glycerol

Table 13. *Fermentation of oxaloacetate and glycerol in various strains of propionic acid bacteria*

30°; 0·045 M $NaHCO_3$; gas phase CO_2; the data refer to 3 ml. suspension.

Strain	mg. cells in 3 ml. suspension	Substrates added (final conc.)	Period of incubation min.	µl. CO_2 formed (free CO_2 from substrate plus CO_2 from bicarbonate)	µl. succinate formed
P. Freudenreichii	28	—	120	56	—
	28	0·0133 M oxaloacetate	120	215	220
	28	0·0133 M oxaloacetate, 0·1 M glycerol	120	675	251
	28	0·1 M glycerol	120	120	77
P. Jensenii	40	—	160	102	59
	40	0·0133 M oxaloacetate	160	561	571
	40	0·0133 M oxaloacetate, 0·1 M glycerol	160	1380	846
	40	0·1 M glycerol	160	301	164
P. Shermanii	30	—	240	147	33
	30	0·0133 M oxaloacetate	240	458	440
	30	0·0133 M oxaloacetate, 0·1 M glycerol	240	1960	1150
	30	0·1 M glycerol	240	227	61
P. Thönii	27	—	240	155	—
	27	0·0133 M oxaloacetate	240	257	49
	27	0·0133 M oxaloacetate, 0·1 M glycerol	240	970	96
	27	0·1 M glycerol	240	407	76
P. rubrum	27	—	240	116	—
	27	0·0133 M oxaloacetate	240	319	25
	27	0·0133 M oxaloacetate, 0·1 M glycerol	240	1025	81
	27	0·1 M glycerol	240	312	87

occur generally in propionic acid bacteria. Five strains of the American Collection of Type Cultures were tested and on the whole the results (Table 13) are similar to those obtained with *P. Shermanii*. In each case oxaloacetate is reduced to succinate, and glycerol increases the rate of succinate formation. The rate of acid production from glycerol was also increased by the addition of oxaloacetate. In the case of *P. Thönii* and *P. rubrum* the yield of succinate was low, and a further investigation of this difference showed that fumarate and malate appear instead of succinate in these strains (Table 14).

Table 14. *Fermentation of oxaloacetate and glycerol in* P. Thönii

39 mg. cells in 3 ml. 0·05 M $NaHCO_3$; CO_2; 5 hr. For the calculation of fumarate + $l(-)$-malate it was assumed that the ratio $\frac{l(-)\text{-malate}}{\text{fumarate}} = 3{\cdot}54$ for temp. 30°.

Substrates added	0·027 M oxaloacetate	0·027 M oxaloacetate 0·1 M glycerol	0·1 M glycerol
Total CO_2 evolved (µl.)	629	2360	700
Succinate formed (µl.)	45	78	119
Fumarate formed (µl.)	78	141	89
Fumarate + $l(-)$-malate (calc.)	354	640	404

Discussion

The evidence in support of scheme (2). The most important part of the evidence in favour of scheme (2) is the simultaneous formation of fumarate and succinate from glycerol (Tables 5 and 14). Since the reaction succinate → fumarate does not occur under anaerobic conditions (Table 10), fumarate formed anaerobically cannot have arisen from succinate: if the formation of succinate by reduction is rejected, the improbable assumption of two separate mechanisms for the formation of succinate and of fumarate must be made.

The fact that all the reactions postulated by (2) can occur, lends much further support to the scheme, for if an added substance is readily metabolized, it is very likely to be a normal metabolite.

Biological significance of the synthesis of oxaloacetate. As shown by Wood & Werkman [1938] glycerol is fermented in propionic acid bacteria in two ways:

$$CH_2OH.CHOH.CH_2OH \longrightarrow CH_3.CH_2.COOH + H_2O,$$
$$CH_2OH.CHOH.CH_2OH + CO_2 \longrightarrow COOH.CH_2.CH_2.COOH + H_2O.$$

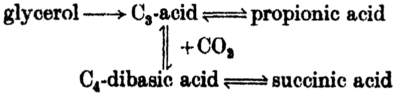
The significance of the second form of glycerol fermentation lies probably in the fact that it produces the oxaloacetate and fumarate required as hydrogen carriers.

It would be of interest to compare the changes of free energy in the two fermentations, but it appears that the necessary data are not yet available.

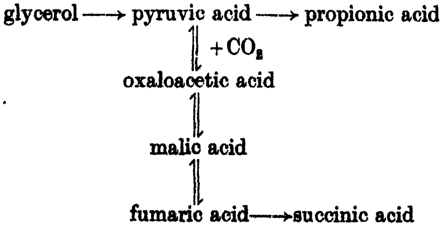
The experiments of Carson & Ruben with radioactive carbon. Carson & Ruben [1940] allowed propionic acid bacteria to ferment glycerol in the presence of CO_2 containing the radioactive isotope C^{11}. The isotope was found to be present in succinic acid and in propionic acid. Its presence in propionic acid was somewhat unexpected and the authors first thought it might be due to the following reversible reaction:

$$CH_3.CH_2.COOH + CO_2 \rightleftharpoons COOH.CH_2.CH_2.COOH.$$

However Carson & Ruben, like ourselves, were unable to find evidence in support of this reaction and they proposed another scheme which explains all the experimental observations:



If 'pyruvic acid' is substituted for 'C_3 acid' and 'fumaric acid' for 'C_4-dibasic acid' the scheme of Carson & Ruben becomes almost identical with the following scheme comprising only reactions which have been shown to occur:



This scheme accounts for all the facts so far observed. It should be noted that the presence of radioactive carbon in propionic acid is explained if the C_4-

dibasic acid is fumaric acid, but it is not explained if it is malic acid. For malic acid, formed by reaction (2), would on breakdown yield a pyruvic acid containing no isotope. On the other hand, fumaric acid containing an active carbon atom in the carboxyl group would be converted into two different malic acids, viz. *COOH.CHOH.CH_2.COOH and COOH.CHOH.CH_2.*COOH (the asterisk denotes the isotope). On breakdown the first would yield a pyruvic acid (and on reduction of the latter a propionic acid) containing the isotope, whilst the second would lose the isotope.

The appearance of the isotope in the propionic acid formed from glycerol may be taken as further evidence in support of the view that the system oxaloacetate ⇌ fumarate acts as a hydrogen carrier in the fermentation of glycerol. It is direct proof of the reversibility, under 'normal' conditions, of the reactions of the scheme (2) leading from pyruvate and CO_2 to fumarate.

SUMMARY

1. Propionic acid bacteria readily reduce oxaloacetate to succinate. Six different strains were examined. Fumarate and *l*(−)-malate are formed as intermediate metabolites. The hydrogen required for the reduction of oxaloacetate can be supplied by several reactions, e.g. the conversion of pyruvate into acetate.

2. Fumarate and *l*(−)-malate are readily reduced to succinate in *P. Shermanii*.

3. A powerful fumarase is present in *P. Shermanii*.

4. The rates of succinate formation from oxaloacetate, fumarate and malate are sufficiently high to allow the assumption that these acids are intermediates in the formation of succinate from glycerol, in accordance with scheme (2).

5. Fumarate is shown to be formed when glycerol is anaerobically fermented by *P. Shermanii*. This is considered as evidence in support of (2).

6. Fumarate, malate or oxaloacetate catalytically accelerate the fermentation of glycerol. It is concluded that these acids act as intermediary hydrogen carriers in the fermentation of glycerol. It is pointed out that the experiments of Carson & Ruben with radioactive carbon support this view.

7. Similar catalytic effects of oxaloacetate were observed when mannitol, inositol, arabinose or erythritol were the substrates of fermentation.

8. The view that succinate is synthesized in propionic acid bacteria from 2 mol. of acetate is rejected. Acetate can be oxidized aerobically, and probably anaerobically, to form CO_2. The oxidative conversion of acetate into CO_2 explains the low yields of acetate observed by previous investigators.

9. The facts concerning the synthesis of succinate in propionic acid bacteria are all explained by scheme (2), according to which the synthesis of oxaloacetate from pyruvate and CO_2 is the primary step in the synthesis of the 4 carbon chain.

This work was aided by grants from the Rockefeller Foundation and from the Medical Research Council.

REFERENCES

Carson & Ruben (1940). *Proc. nat. Acad. Sci., Wash.*, **26**, 418.
Erkama (1938). *Suomen Kemistilehti*, **11** G, 28 (quoted from *Chem. Abstr.* (1939), p. 5023).
Fitz (1878). *Ber. dtsch. chem. Ges.* **11**, 1890.
Fromageot & Bost (1938). *Enzymologia*, **4**, 225.
Gladstone & Fildes (1940). *Brit. J. exp. Path.* **21**, 61.
Jensen (1931). Dairy Bacteriology. London.
Krebs & Eggleston (1940). *Biochem. J.* **34**, 1383.
—— —— Kleinzeller & Smyth (1940). *Biochem. J.* **34**, 1234.
—— Smyth & Evans (1940). *Biochem. J.* **34**, 1041.
Musicant & Kaszuba (1939). *J. Amer. chem. Soc.* **61**, 2974.
Quastel & Webley (1939). *Nature, Lond.*, **144**, 633.
—— —— (1941). *Biochem. J.* **35**, 192.
Sherman (1920). *J. Bact.* **6**, 379.
Stone, Wood & Werkman (1936). *Biochem. J.* **30**, 624.
van Niel (1928). The Propionic Acid Bacteria. Haarlem.
Warburg & Yabusoe (1924). *Biochem. Z.* **146**, 380.
Werkman & Brown (1933). *J. Bact.* **26**, 393.
Wood & Werkman (1936, 1). *Biochem. J.* **30**, 481.
—— —— (1936, 2). *Biochem. J.* **30**, 618.
—— —— (1938). *Biochem. J.* **32**, 1362.
—— —— (1940). *Biochem. J.* **34**, 7.
—— —— Hemingway & Nier (1940). *J. biol. Chem.* **135**, 789.
—— Stone & Werkman (1937). *Biochem. J.* **31**, 349.

72. CYTOCHROME SYSTEM IN *BACTERIUM COLI COMMUNE*

BY D. KEILIN AND C. H. HARPLEY

From the Molteno Institute, University of Cambridge

(*Received 25 May 1941*)

THE aerobic respiration of *Bact. coli*, like that of *Bacillus subtilis*, baker's yeast and other aerobic cells, is strongly and reversibly inhibited by KCN, H_2S, NaN_3 and CO, but, unlike that of *B. subtilis* or cells of baker's yeast, the inhibition with CO is not light-sensitive.

The absorption spectrum of cytochrome in *B. coli* differs markedly from that of *B. subtilis* or yeast cells. Thus, the band *a* is absent from *B. coli*, and is replaced by a hardly perceptible shading a_1 at 590 mμ and a feeble band a_2 at 628 mμ which is similar to the band a_2 of *Azotobacter*. This band in organisms exposed to CO moves to 634 mμ, on oxidation it is shifted to 645 mμ and in presence of KCN and O_2 it disappears completely[1] [Keilin, 1934; Fugita & Kodama, 1934]. The band a_2 belongs therefore to an autoxidizable haemochromogen compound which reacts with KCN and with CO. According to Lemberg & Wyndham [1937] this compound can be considered as a bile pigment haemochromogen with an open tetrapyrrolic chain kept together in a ring form by the central iron atom. Lemberg ascribes to a_2 the structure of a biliviolin-haemochromogen which is obtained by dehydrogenation of verdohaemochromogen. The component a_2 appears therefore to belong to a bile pigment derivative of haematin *a*, and, as in all the derivatives with an open tetrapyrrolic chain [Holden & Lemberg, 1939], its absorption spectrum is devoid of the Soret or γ-band.

The properties of a_2, namely its reactions with O_2, CO and KCN, suggest that in *B. coli*, as in *Azotobacter* [Negelein & Gärischer, 1934], this component may act as cytochrome oxidase. On the other hand, the failure to find any parallelism between the concentration of a_2 and the respiratory activity of cells containing this component leaves open the possibility of the existence in *B. coli* of yet another autoxidizable haematin compound acting as cytochrome oxidase and capable of oxidizing the component b_1.

The bands *b* and *c* of a typical cytochrome are replaced in *B. coli* by one band which lies at about 560 mμ and which represents the α-band of a non-autoxidizable haemochromogen compound b_1. This band is asymmetric and shows on its short-wave side a reinforcement which, according to Yamagutchi [1937], may represent the band *c* masked by an extension of the band b_1.

On freezing and cooling the suspension of *B. coli* to the temperature of liquid air, the short-wave side of the band b_1 becomes greatly intensified and appears at 551 mμ united by a shading to a much fainter band at 559 mμ. The α-band of cytochrome *c* which, under similar conditions, is also markedly intensified is, however, shifted from its usual position at 549·5 to 547 mμ. This clearly shows, even if we assume that the asymmetric band b_1 of *B. coli* is composed of two fused bands belonging to two distinct compounds, that neither of them can be identified with the true components *b* and *c* of cytochrome.

---

[1] In some other strains of *B. coli* and in *Proteus vulgaris* Yamagutchi [1937] failed, however, to detect any reaction between a_2 and KCN.

The component b_1 in living intact cells of *B. coli* does not react with KCN, NaN_3 or CO, which however inhibit its oxidation.

Catalytic activity of cytochrome in Bact. coli

Unlike *B. subtilis* and many other aerobic cells, *B. coli*, even when free from metabolites, is incapable of oxidizing *p*-phenylenediamine or 'Nadi' reagent [Happold, 1930]. This may be partly explained by the absence from *B. coli* of cytochrome *c* which, as was previously shown [Keilin & Hartree, 1938], is essential for these catalytic oxidations. A washed suspension of *B. coli* can neither reduce nor oxidize added cytochrome *c*, which is not surprising considering that cytochrome *c*, being a comparatively large molecule, cannot diffuse through the cell wall of *B. coli* and thus come in contact with its intracellular oxidizing systems. The direct reaction between cytochrome *c* and the oxidizing systems of *B. coli* can only be determined when crushed micro-organisms, in which these systems are exposed, are brought into contact with a solution of cytochrome *c*.

The study of this inter-reaction was carried out in a series of spectroscopic and manometric experiments. For this purpose *B. coli* were grown in the usual way on a peptone-agar medium[1] in about thirty large Roux flasks. The organisms after 24 hr. growth were collected, washed several times in saline solution and mixed with 30 ml. of phosphate buffer solution at *p*H 7 to a thick suspension which was ground for about 1 hr. in a Booth & Green [1938] bacterial mill. The ground preparation thus obtained gave on centrifuging three distinct layers: the top layer (*A*) of opalescent fluid, the intermediate or granular layer (*B*) composed of crushed bacteria and the bottom layer (*C*) of intact bacteria. The first two layers (*A* and *B*), collected separately and dialysed for at least 14 hr. against a large amount of 0·02 *M* phosphate buffer solution (*p*H 7·3), were found to possess similar properties, except that the fraction *B* displayed a stronger absorption spectrum and a greater catalytic activity than fraction *A*. The fraction *B*, which was used for most of the observations recorded below, did not take up O_2 and its cytochrome remained in a completely oxidized state. If, however, substrates such as succinic, lactic or formic acids were added to this preparation, they became rapidly oxidized and the preparation took up O_2 vigorously. Cytochrome in such preparations shaken with air undergoes rapid oxidation and reduction.

As all the components of the oxidizing systems in this preparation are now exposed they can easily be brought into contact with cytochrome *c*.

Spectroscopic experiments

For spectroscopic study, a small amount of pure cytochrome *c* prepared from heart muscle as previously described [Keilin & Hartree, 1937] was added to fractions *A* or *B* of crushed bacteria in presence and in absence of substrate. Spectroscopic examination of these mixtures showed that the dialysed fractions (*A* or *B*) of crushed *B. coli*, unlike similar preparations of baker's yeast, do not oxidize reduced cytochrome *c* even when the mixtures are vigorously shaken with air. The same preparations in the absence of substrate do not reduce oxidized *c*. They reduce this compound, however, in the presence of substrates such as succinic or lactic acids. Once reduced, cytochrome *c* does not undergo reoxidation on shaking the mixture with air, while components a_2 and b_1 of cytochrome proper to *B. coli* undergo normal and rapid reoxidation.

---

[1] The nutrient medium is composed of 20 g. peptone, 20 g. 'Lab. Lemco,' 10 g. NaCl, 60 g. agar and 2 l. water.

Manometric experiments

It has been shown [Keilin, 1930; Keilin & Hartree, 1938] that the addition of cytochrome *c* to enzyme preparations obtained from heart muscle, liver or kidney, greatly increases the rates of oxidation of different substances, such as cysteine, succinic acid, *p*-phenylenediamine or adrenaline, catalysed by these preparations. The addition of cytochrome *c* increases also the rates of oxidation of lactic acid or of *p*-phenylenediamine by enzyme preparations obtained from crushed cells of baker's yeast.

Similar experiments were carried out in Barcroft differential manometers at 39° with both fractions (*A* and *B*) of the preparation obtained from crushed *B. coli*. The enzyme preparations were suspended in 3 ml. *M*/15 phosphate buffer, *p*H 7·3. The substrates, such as succinic or lactic acids, or *p*-phenylenediamine, were added from dangling tubes which were dislodged only after the temperature of the manometers had been equilibrated and the taps closed. The final concentrations of substrate were *M*/50. The clear fraction (*A*) was used in these experiments in a higher concentration than the granular and more active fraction (*B*). In some experiments the enzyme preparation received 0·2 ml. of 1 % cytochrome *c*, or 10^{-3} *M* methylene blue and cyanide.

Table 1

B. coli enzyme prep. ml.	Cytochrome *c*	*p*-phenylenediamine	Succinic acid	Lactic acid	KCN	M.B.	O_2 uptake µl. in 25 min.
0·5 (*A*)	–	+	–	–	–	–	6
+	+	+	–	–	–	–	6
+	–	–	+	–	–	–	174
+	+	–	+	–	–	–	160
+	–	–	+	–	+	–	17
+	–	–	+	–	+	+	66
+	–	–	–	+	–	–	215
+	+	–	–	+	–	–	212
0·1 (*B*)	–	+	–	–	–	–	13
+	+	+	–	–	–	–	16
+	–	–	+	–	–	–	198
+	+	–	+	–	–	–	160
+	–	–	–	+	–	–	166
+	+	–	–	+	–	–	164
+	–	–	–	+	+	–	11
+	–	–	–	+	+	+	174

The results of these experiments, which are summarized in Table 1, show that the oxidation of succinic or lactic acid which is actively catalysed by preparations of crushed *B. coli* is not accelerated by the addition of cytochrome *c*. Under similar conditions cytochrome *c* greatly increases the rate of catalytic oxidation of these substances by crushed cells of baker's yeast. Moreover, crushed *B. coli*, even on addition of *c*, oxidized neither *p*-phenylenediamine nor other artificial chromogens such as adrenaline, Nadi reagent, quinol or catechol. The main conclusion which can be drawn from these experiments is that cells of *B. coli* contain only the systems which are capable of reducing cytochrome *c* but are completely devoid of a catalyst capable of oxidizing the reduced *c*. Such a catalyst is, however, present in cells containing the typical cytochrome system. It was shown recently [Keilin & Hartree, 1939] that these cells, in addition to components *a*, *b* and *c* of cytochrome, contain also a fourth component described as a_3. This component is thermolabile and autoxidizable; it forms reversible compounds with KCN, H_2S, NaN_3 and CO and the absorption bands of its CO compound lie at about 591 and 430 mμ.

It was also shown that the catalytic activities of a tissue preparation measured by the velocity of oxidation of *p*-phenylenediamine in presence of an excess of *c* appear to depend on the concentration of the component a_3. This component seems, therefore, to possess all the properties of cytochrome oxidase and directly or indirectly must be responsible for the catalytic oxidation of cytochrome *c*. It is now conceivable that the failure of *B. coli* to oxidize cytochrome *c* is due to the absence from these micro-organisms of the component a_3 which invariably accompanies the cytochrome *a*.

The results obtained for *B. coli* will probably apply to cells of some other micro-organisms (bacteria and yeast) as well as some cells of higher organisms, in which the cytochrome system is devoid of components *a*, a_3 and *c*.

The fact that cytochrome *c* cannot act as a carrier between the dehydrogenases of *B. coli* and their cytochrome oxidase does not exclude the possibility that it may act as a link uniting into an artificial system the dehydrogenases of *B. coli* with cytochrome oxidase of heart muscle. To test such a possibility, the rates of oxidation of lactic and formic acids by the enzyme preparation obtained from crushed bacteria were determined in presence and absence of cytochrome *c* and of 0·1 ml. of a fresh heart-muscle preparation, which can rapidly oxidize the reduced *c*. The results of these experiments, summarized in Table 2, clearly show that the rates of oxidation of lactic and formic acids by the enzyme preparation obtained from crushed *B. coli* are not affected by the addition of cytochrome *c* and cytochrome oxidase prepared from heart muscle. The substrate molecules, activated by their specific dehydrogenases, appear to react with molecular O_2 only through the cytochrome system proper to *B. coli* and not through the cytochrome system of heart muscle added to the *B. coli* preparation. These results suggest that the components of each of the insoluble dehydrogenase systems, together with those of cytochrome, are intimately bound to the protein of a single colloidal particle, forming thus a unit of a complete oxidizing system. The efficiency of such a system is naturally assured by the mutual accessibility of its components, which depends on their spatial distribution and orientation. This view explains the different degree of inhibition by CO of the oxidation by resting *B. coli* of succinic, lactic and formic acids [Cook *et al.* 1931] without postulating the existence of three different cytochrome oxidases. In fact the three different values of *K* (partition constant of oxidase between O_2 and CO) obtained by these workers in their experiments with the oxidation of these

Table 2

B. coli enzyme 0·05 ml. (*B*)	Cytochrome *c*	Cytochrome oxidase 0·1 ml.	Lactate	Formate	O_2 uptake μl.
+	−	−	+	−	136
+	+	−	+	−	156
+	+	+	+	−	174
+	+	+	−	−	50
+	−	−	−	+	140
+	+	+	−	+	123
+	−	−	+	−	168
+	+	+	+	−	138
+	+	+	+	+	285

three substances may simply be due to the different degree of accessibility between each of the dehydrogenases and the components of cytochrome bound in the same colloidal particle. The efficiency of the oxidizing systems of the cell and the co-ordination of the multiple reactions simultaneously proceeding within the cell must therefore depend not only on the integrity of all the components of these systems but also on that of the colloidal structure of the cell which supports them and regulates their mutual accessibility.

SUMMARY AND CONCLUSIONS

1. Contrary to the view of Yamagutchi, *Bact. coli* are completely devoid of cytochrome *c*.

2. The preparations obtained from crushed *B. coli* in which all the oxidizing systems are exposed are not capable of oxidizing reduced cytochrome *c* added to them. *B. coli* and probably some other cells are completely devoid of systems or catalysts capable of oxidizing cytochrome *c*.

3. It is therefore not surprising that the preparations of crushed *B. coli*, even on addition of cytochrome *c*, oxidize neither *p*-phenylenediamine nor other chromogens and that the oxidation of succinic and lactic acids by these preparations is not accelerated by the addition of *c*.

4. The failure by *B. coli* to oxidize cytochrome *c* is due to the absence from the cells of this micro-organism of the component a_3 which invariably accompanies cytochrome *a* and which directly or indirectly is responsible for the oxidation of cytochrome *c*.

REFERENCES

Booth & Green (1938). *Biochem. J.* **32**, 885.
Cook, Haldane & Mapson (1931). *Biochem. J.* **25**, 534.
Fugita & Kodama (1934). *Biochem. Z.* **273**, 186.
Happold (1930). *Biochem. J.* **24**, 1737.
Holden & Lemberg (1939). *Aust. J. exp. Biol. med. Sci.* **17**, 133.
Keilin (1930). *Proc. roy. Soc.* B, **106**, 418.
—— (1934). *Nature, Lond.*, **133**, 290.
—— & Hartree (1937). *Proc. roy. Soc.* B, **122**, 298.
—— —— (1938). *Proc. roy. Soc.* B, **125**, 171.
—— —— (1939). *Proc. roy. Soc.* B, **127**, 167.
Lemberg & Wyndham (1937). *J. roy. Soc. N.S.W.* **70**, 343.
Negelein & Gärischer (1934). *Biochem. Z.* **268**, 1.
Yamagutchi (1937). *Bot. Mag., Tokyo*, **51**, 457.

73. VITAMIN A STUDIES WITH RATS AND PIGS

By R. BRAUDE, A. S. FOOT, K. M. HENRY, S. K. KON,
S. Y. THOMPSON AND T. H. MEAD

From the National Institute for Research in Dairying, University of Reading, and the Research Laboratories, The British Drug Houses Ltd., London, N. 1

(*Received 3 May 1941*)

MANY aspects of the metabolism of vitamin A and of its precursors are still insufficiently known; this is especially true with regard to information available for animals other than the rat. Two of us (A. S. F. and S. K. K.) have for some time been interested in the importance of vitamin A in pig feeding. For reasons both practical and scientific we wanted to gain more knowledge of the utilization by the pig of certain carotenoids, of the relative value for the pig of carotene and of preformed vitamin A and of the minimum vitamin A requirements of this animal. Another of the authors (T. H. M.) has studied on rats the biological activity of vitamin A alcohol and of natural vitamin A esters and further comparisons using a different animal were thought likely to be of value. The present joint paper gives the results of all these inquiries. The biological tests were carried out at Shinfield and the chemical and physical manipulations were mostly done in the laboratories of The British Drug Houses, Ltd.

It is generally accepted that in order to exhibit vitamin A activity a carotenoid pigment must contain a β-ionone ring or rings. Recently, however, Lund [1938] and Petersen [1939] reported that zeaxanthin was effective in preventing symptoms of vitamin A deficiency in pigs, but that 'xanthophyll' (lutein) was inactive in this respect. If confirmed, this finding would be of considerable importance, not only because it would demonstrate the unique ability of the pig to convert a dihydroxycarotene into vitamin A, but also from the practical standpoint of pig feeding. Maize, an important pig food, contains relatively more zeaxanthin than carotene and cryptoxanthin [Kuhn & Grundman, 1934]. The observation of Lund [1938] that the pig can make use of dihydroxy-β-carotene but not of dihydroxy-α-carotene (lutein, xanthophyll) is certainly puzzling, as it implies that a hydroxy-β-ionone ring is utilized in one case but not in the other. The preparation of zeaxanthin used by Lund [1938] was admittedly contaminated with carotene, and we felt that for this and other reasons a re-investigation of the value of zeaxanthin as a source of vitamin A for pigs was amply justified.

Pure β-carotene has, by definition, a potency of $1{\cdot}67 \times 10^6$ I.U./g., while, according to most recent data [Holmes & Corbett, 1937; Mead *et al.* 1939; Baxter & Robeson, 1940], the potency of pure vitamin A measured on the rat is nearly double this figure, i.e. about $3{\cdot}0$–$3{\cdot}3 \times 10^6$ I.U./g. These figures indicate that this animal, even under most favourable conditions, can form only one molecule of vitamin A from one molecule of β-carotene. This is what one would expect on theoretical grounds, as there is no reason to suppose that the fission of the β-carotene molecule takes place symmetrically [Morton, 1940].

The information regarding other animals is less definite, but Guilbert *et al.* [1940] have shown that cattle, sheep, pigs and horses are, if anything, less efficient than rats in converting carotene into vitamin A. Full references to this work will be found in a recent paper by Hart [1940]. It was found that to cover minimum requirements the four species needed 5·3–7·1 μg. of vitamin A[1] per kg. body weight but that 24–34 μg. of carotene, either from artificially dried lucerne or as a preparation (88 % β- and 12 % α-carotene) in oil, were required for the same purpose. The animals obtained therefore a yield of only about half a molecule of vitamin A from one molecule of β-carotene. At least 5–10 times the minimum level is recommended in practice by the American authors. In this country, Dunlop [1934] found that 14 mg. of carotene per 100 lb. of food are insufficient for normal growth of pigs, while 62 mg./100 lb. give normal growth with storage of the vitamin. These values correspond for a 50 kg. pig to 12 and 53 μg. respectively of carotene per kg. body weight [Dunlop, 1933] and agree with the American experience. According to Lund [1938] the minimum requirement of pigs up to 40 kg. in weight does not exceed 30 μg./kg. body weight.

The relation between the biological activity of materials containing vitamin A and their spectral absorption at 328 mμ remains uncertain in spite of the numerous investigations that have been devoted to this subject [e.g. Coward *et al.* 1931; 1932; Morgan *et al.* 1935; Hume, 1937; 1939; Moll & Reid, 1939]. Values ranging from below 1000 to over 3000 have been found for the factor for converting $E^{1\%}_{1\,\text{cm.}}$ 328 mμ to biological units, and statistical analyses have in some cases [Hume, 1939; Morgan *et al.* 1935] indicated that the differences between the factors assigned to different materials are greater than the variations to be expected on account of the errors inherent in biological tests.

Although a survey of the available results reveals no hard and fast relation between the type of material tested and its appropriate conversion factor, certain generalizations hold with few exceptions. Some commercial vitamin A concentrates prepared from fish liver oils by saponification tend to give low factors, as also do mammalian liver oils and the corresponding concentrates. Higher values are usually given by unhydrolysed fish liver oils and in particular by fresh medicinal cod-liver oils and by the reference oil of the United States Pharmacopoeia. Exceptionally high factors have been claimed by Moll & Reid [1939] for samples of a commercial vitamin A concentrate which is prepared without saponification.

A number of explanations for the wide variation in experimentally determined conversion factors have been suggested. Pritchard *et al.* [1937] have, for example, shown that mammalian liver oils contain a factor which contributes to spectral absorption at 328 mμ but has little biological activity. The presence of this material probably accounts for the low conversion factors found for mammalian liver oils. The low biological activity of some fish liver oils relative to their extinction coefficient at 328 mμ may similarly be due to the presence of oxidized vitamin A [Robinson, 1938], cyclized vitamin A [Edisbury *et al.* 1932; Embree, 1939], vitamin A_2, or possibly other substances having little or no biological activity. Another explanation is that different oils may contain different proportions of the many possible isomers of vitamin A.

Concentrates prepared by saponification have most frequently been found to give the lowest conversion factors and it is therefore conceivable that this operation affects the potency of the vitamin. Since the vitamin A in fish liver oils is esterified, while that in concentrates prepared by saponification is free

---

[1] Wherever quantities of vitamin A are in this paper expressed in terms of weight it has been assumed that pure vitamin A has $E^{1\%}_{1\,\text{cm.}}$ 328 mμ = 1800 [Mead *et al.* 1939; Baxter & Robeson, 1940]. Quantities quoted from the literature have been recalculated to this basis to facilitate comparison.

[Bacharach & Smith, 1928; Reti, 1935; Hamano, 1935; Hickman, 1937; Tischer, 1938], the question arises as to whether a lower conversion factor should be ascribed to vitamin A alcohol than to vitamin A esters.

The existing evidence consists for the most part in experiments in which crude fish oils and crude concentrates were assayed against β-carotene on rats. In 1936 careful assays of a sample of halibut liver oil and of its non-saponifiable fraction were carried out in ten laboratories. Although the results [Hume, 1937; 1939] showed a higher conversion factor for the oil than for the concentrate, the latter was found by the spectrographic test to have deteriorated during the test period by an amount sufficient to account for the discrepancy. Emmet & Bird [1937] reported that tests carried out on a fish liver oil and on concentrates prepared from it by varying degrees of saponification indicated that a lowering of conversion factor occurred which ran parallel with the progress of hydrolysis. Hickman [1939] stated that distilled esters of vitamin A had the same conversion factor as the vitamin A in crude oils, whereas concentrates prepared by saponification always had a smaller factor which might be less than half that of the oils. Moll & Reid [1939], using a bioassay technique differing greatly from that normally used in England, found conversion factors of from 3500 to 3700 for a commercial vitamin A concentrate containing vitamin A esters. To the non-saponifiable fraction of this concentrate they assigned a factor of 1800.

The problem under discussion is complicated by the following considerations: (*a*) vitamin A alcohol in concentrates is more readily oxidized than vitamin A esters present in fish liver oils; (*b*) oxidation products of vitamin A absorb strongly in the neighbourhood of 328 mμ but have little or no biological activity [Edisbury *et al.* 1932; Robinson, 1938]. It is therefore to be expected that low conversion factors will be found for commercial vitamin A concentrates which may have become partially oxidized during preparation and storage, as well as for concentrates which have been carefully prepared in the laboratory but which have not been fully protected against oxidation during the bioassay period. It is evident that the conversion factor applicable to any material containing vitamin A depends in the first place on its content of impurities affecting the spectrographic test. It follows that evidence of the true relation between the biological activities of free and esterified vitamin A is obtainable only from tests on materials free from such impurities.

One object of the present experiment was to discover whether any difference could be detected between the potencies of vitamin A alcohol and natural vitamin A esters when both had been freed as completely as possible from spectrographic impurities by molecular distillation. Simultaneous tests of the purified materials were carried out on pigs and on rats. A more comprehensive comparison of similarly purified materials was carried out on rats by Dr Underhill at The British Drug Houses, Ltd., before the experiment on pigs was undertaken. His results will form the subject of a separate paper.

EXPERIMENTAL

Preparation of materials

Vitamin A alcohol and purified natural vitamin A esters. Our principal object in the purification of these materials was to eliminate as completely as possible substances such as oxidized and cyclized vitamin A which give rise to irrelevant absorption at 328 mμ.

Vitamin A alcohol having $E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ = 1600 was used for these experiments. It was prepared as described by Mead *et al.* [1939] by molecular distillation of the non-saponifiable fraction from a rich fish liver oil.

The natural vitamin A esters were made by purifying an ester concentrate obtained by distilling unhydrolysed fish liver oil. The crude material, a dark brown semi-solid (Carr-Price value 21,000), was dissolved in light petroleum and the solution was filtered through a column of activated alumina. The column was rinsed with the solvent until the brown zone, which at first occupied the top half, reached nearly to the bottom. The filtrate and rinsings were concentrated under diminished pressure, leaving a pale yellow oil which was distilled in a cyclic molecular still of the Hickman type. A first fraction consisting of a small quantity of red oil containing some cyclized vitamin A and vitamin A alcohol was obtained by circulating the distilland over the distilling surface successively at 80°, 90°, 100°, 120°, 130°, 140°, 150° and 155°. A second fraction consisting of vitamin A esters contaminated with sterols and traces of vitamin A alcohol was removed by cycles at 160°, 165° and 170°. The third fraction, which was used to prepare the feeding solutions for this experiment, was collected by circulation once at 175° and twice at 180°. It consisted of a pale yellow oil having Carr-Price value 33,000 and $E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ = 660.

If pure vitamin A alcohol has an extinction coefficient $E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ = 1800, this concentrate of the natural esters contained 36·7 % of vitamin A. On the assumption that the average molecular weight of the fatty acids combined with the vitamin A is of the same order as that of oleic acid, the vitamin A ester concentrate contained about 73 % of natural vitamin A esters. The remainder probably consisted chiefly of fatty triglycerides and fatty acid esters of cholesterol.

β-Carotene. A specimen of commercial crystalline mixed carotenes was purified by chromatographic adsorption on magnesia followed by crystallization from benzene-ethanol. At no time during the preparation was the substance exposed to a temperature exceeding 45°. The product had M.P. 184° (corr.) and $E^{1\%}_{1\,\mathrm{cm}}$ 456 mμ = 2400 (in cyclohexane).

Zeaxanthin was obtained by hydrolysis of the naturally occurring dipalmitate (physalien). The hydrolysate was carefully separated by chromatography from carotene, cryptoxanthin and other carotenoids present in crude extracts of *Physalis*. The seed pods from freshly picked *Physalis* (approx. 30 kg.) were separated from the berries, covered immediately with alcohol and set aside for a month. The alcohol was run off and the pods were air-dried, first at room temperature and finally overnight at 37°, before being pulverized in a ball-mill from which the air had been displaced by CO_2. The powder was immediately extracted with successive portions of light petroleum until all the pigment had been removed. The combined extracts were evaporated in an atmosphere of N_2 under diminished pressure to a small bulk; sufficient benzene was added to give a clear solution (vol. approximately 1·5 l.), which was treated with alcohol (4·5 l.). After a short time, the crude physalien was collected, washed with alcohol, and immediately recrystallized by the addition of warm alcohol to its solution in warm benzene. Yield 57 g. The physalien was hydrolysed and the crude zeaxanthin purified in several small batches as follows.

Physalien (12·5 g.) was dissolved in benzene (125 ml.). The solution was diluted with light petroleum (1875 ml.) and shaken for 8 hr. at room temperature with a solution of KOH (150 g.) in methyl alcohol (625 ml.). On the addition of distilled water (about 1 l.) the zeaxanthin separated at the interface between the petroleum and aqueous-methanol phases. The upper and lower layers were siphoned off leaving the zeaxanthin suspended in a small volume of liquid from which it was separated by filtration. The product was thoroughly washed on the filter with 50 % methyl alcohol and dried in a desiccator. Yield 6·5 g. from 12·5 g. physalien. For purification the crude material was dissolved in warm ethylene dichloride (3·5 l.) and the solution, after being cooled to room temperature, was filtered through a column (ca. 11 × 55 cm.) containing 3 kg. of 'heavy' magnesia. The chromatogram was developed with ethylene dichloride (2·5 l.) followed by ethylene dichloride mixed with 15 % benzene (1 l.) until the intermediate orange band containing the zeaxanthin was well separated from the zones above and below it. The appropriate zone was dissected out and eluted with chloroform containing methyl alcohol (5 %). The eluate was washed several times with water in order to remove methyl alcohol, dried (Na_2SO_4) and evaporated under diminished pressure to a small volume from which the zeaxanthin crystallized on the addition of light petroleum. After recrystallization from chloroform (150 ml.) the product melted at 214° (uncorr.). Yield 3·35 g. A solution in ethanol showed absorption bands at 454 and 480 mμ:

$$E^{1\%}_{1\,\mathrm{cm}}\ 454\ \mathrm{m}\mu = 2520. \quad E^{1\%}_{1\,\mathrm{cm}}\ 480\ \mathrm{m}\mu = 2230.$$

Strain [1938] gives the following data for pure zeaxanthin (M.P. 212–213°). Absorption bands (in ethanol) at 423·5 (weak), 451·5 and 483 mμ. The following extinction coefficients are taken from the curve on p. 102 of Strain's monograph:

$$E^{1\%}_{1\,\mathrm{cm.}}\ 454\ \mathrm{m}\mu = 2470. \quad E^{1\%}_{1\,\mathrm{cm.}}\ 480\ \mathrm{m}\mu = 2190.$$

Preparation and storage of the animal feeding solutions

Solvent oil. Arachis oil specially selected on account of low peroxide content and the negligible absorption given at 328 mμ was used for preparing the test solutions after treatment with quinol (0·01 %).

Solutions of vitamin A alcohol. In calculating the quantities to be used for making the solutions of vitamin A alcohol and vitamin A esters, it was assumed that the potency in I.U./g. is equal to the extinction coefficient multiplied by 1600. A solution containing approximately 500 I.U./g. for feeding to the pigs was prepared by dissolving 1·82 g. of vitamin A alcohol ($E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ = 1600) in 541·1 g. of arachis oil and diluting part of this solution (273·7 g.) to 4·87 kg. A solution containing 200 I.U./g. for the rat experiment was prepared from this by appropriate dilution with arachis oil. The extinction coefficients of the two solutions were measured immediately after preparation and again at intervals during the biological experiments. The results of these tests and of those on the solutions of vitamin A esters, β-carotene and zeaxanthin are given in Table 1.

Solutions of vitamin A esters. A solution containing 500 I.U./g. of the purified vitamin A esters in arachis oil was prepared by dilution in two stages in a manner similar to that used for making the vitamin A alcohol solution. From this the 200 I.U./g. solution for the rat experiment was prepared by dilution with the same solvent.

Solutions of β-carotene. The biological activity of the carotene was assumed to be 1,670,000 I.U./g. The following procedure was adopted in order to avoid the risk of decomposing the carotene by exposure to temperatures above 40°. β-Carotene (1·314 g.) was dissolved in pure benzene (300 ml.) and approximately one-third of this solution was added to about 1 l. of arachis oil contained in a 5 l. flask. The greater part of the benzene was removed in a vacuum and the solution transferred to a tared 10 l. flask. These operations were repeated twice, using the remaining two-thirds of the solution of carotene in benzene, the last

Table 1. *Spectrophotometric measurements on the feeding solutions*

Substance	Correct potency I.U./g.	Date	$E^{1\%}_{1\,\text{cm.}}$	I.U./g.
Vitamin A alcohol	500	7. ii. 40	0·303	485
			0·304	
		13. iii. 40	0·276	454
			0·292	
		1. v. 40	0·286	458
		1. viii. 40	0·295	465
			0·286	
	200	8. ii. 40	0·121	194
		12. iii. 40	0·115	185
Vitamin A esters	500	6. i. 40	0·300	488
			0·310	
		13. iii. 40	0·300	480
		1. v. 40	0·297	475
		1. viii. 40	0·271	435
			0·271	
		3. viii. 40*	0·295	473
	200	6. i. 40	0·121	194
		12. iii. 40	0·118	189
β-Carotene	500	3. i. 40	0·710	494
		13. iii. 40	0·750	522
		1. v. 40	0·750	522
		1. viii. 40	0·647	450
	200	6. i. 40	0·290	202
		12. iii. 40	0·285	198
Zeaxanthin	200†			
	1st batch	3. ii. 40	0·320	200
	2nd batch	27. iii. 40	0·305	191
	3rd batch	7. v. 40	0·301	188
	4th batch	6. vi. 40	0·286	179
	5th batch	1. vii. 40	0·303	189
	6th batch	22. vii. 40	0·285	179

* Fresh unopened bottle.

† Presumptive 'units' based on assumption that 1 mol. zeaxanthin has the activity of 1 mol. of β-carotene.

traces of which were rinsed in with a small quantity of the solvent. The 5 l. flask was finally washed several times with arachis oil and the washings were added to the contents of the 10 l. flask so as to bring the total weight of the solution to 4·38 kg. The last traces of benzene were removed by connecting the flask to a vacuum pump of large capacity while a slow stream of CO_2 from a submerged sintered-glass gas distributor passed through the solution. The temperature of the oil was raised to 40° during the later stages of this operation and care was taken to exclude light. A solution containing 200 I.U./g. for feeding to the rats was prepared from the stronger solution by dilution with arachis oil.

Solution of zeaxanthin. It was intended that some of the pigs should be given quite large doses of zeaxanthin in order that biological activity of a low order could be detected. Attempts were therefore made to prepare rather concentrated solutions in the hope that feeding of excess arachis oil to these pigs could be avoided. Owing, however, to the low solubility of zeaxanthin in oil, we were compelled to use a solution no stronger than 0·0128 %. Such a solution would contain 200 I.U./g. if zeaxanthin had the same activity, molecule for molecule, as β-carotene. We discovered later that zeaxanthin crystallized slowly on storage at room temperature even from solutions as dilute as this. The resulting fall in concentration of the six batches of solution made during the course of the experiment was estimated colorimetrically by means of a Leitz photometer at intervals during the period in which each batch was fed to the pigs. The loss, which never exceeded 10 %, was considered to be negligible. Each batch weighed about 8 kg. and was prepared by a method similar to that used for preparing the carotene solution, except that chloroform was used in place of benzene because the latter is a very poor solvent for zeaxanthin. A portion of the first batch was used for the rat test. The batches were prepared as required in order to avoid long periods of storage.

Storage and stability of the feeding solutions. The solutions were kept under N_2 in a refrigerator. In spite of these precautions some deterioration occurred, as is shown in Table 1.

Levels of feeding

All substances tested were given to pigs and rats at two levels of intake. For pigs the low level was based on the estimates of minimum requirements put forward by Hart [1940], but to find out any differences all substances were fed at the same presumptive level of biological activity.

As the lower dose, 100 I.U. of vitamin A were given daily for 10 lb. live weight. This was equivalent to 7·7 μg. of vitamin A (as alcohol or natural esters) and 13·3 μg. of β-carotene per kg. body weight (*vide supra*). The higher dose for these substances was 300 I.U./10 lb. live weight. Zeaxanthin was presumed to be as active as β-carotene but was fed at levels of 300 and 1500 calculated I.U./10 lb. live weight. Details of rat tests are given separately on p. 703.

Tests on pigs

Methods. A 'normal' pig-meal mixture, similar to that used by us previously [Foot *et al.* 1938; 1939], was prepared for this experiment. It consisted of:

Barley meal	40
Weatings	45
Extracted groundnut cake ...	5
Meat meal	6
Brewer's yeast	2
Ground limestone	1½
Common salt	½

Examination showed that the diet contained only traces of yellow pigment and this was mostly xanthophyll. No β-carotene was detected; the carotene content was certainly much less than 0·02 mg. per 100 g.

The general methods of management of the pigs were as already described [Foot *et al.* 1938; 1939].

Three litters of pigs were reared on this ration. The dams of each litter were given the meal shortly after farrowing and received this food alone during suckling. The young pigs were offered 4 parts of the meal mixture together with 1 part of dried skim milk powder from the time they were 3 weeks old; they were weaned when 8 weeks old and the three litters were then pooled, groups from them being selected for five treatments as follows:

Group 1. 2 pigs as controls; no addition to meal mixture.
Group 2. 6 pigs receiving zeaxanthin, 3 the higher and 3 the lower level.
Group 3. 4 pigs receiving β-carotene, 2 the higher and 2 the lower level.
Group 4. 4 pigs receiving vitamin A alcohol, 2 the higher and 2 the lower level.
Group 5. 4 pigs receiving vitamin A ester, 2 the higher and 2 the lower level.

The grouping was carried out in such a way that the litters were as far as possible evenly distributed among treatments. In Fig. 1 and Tables 2 and 3 littermates are indicated by letters.

After grouping, the addition of skim-milk powder was discontinued and the meal alone was fed. Each pig was given in addition once a fortnight a solution of calciferol in oil supplying 500 I.U. of vitamin D daily. The pigs were group-fed twice daily. The amount of food allowed each pen of pigs was based on the live weight of each animal and was adjusted once a week after the animals had been weighed. The standard used was that usually recommended by the Pig Experiments Coordination Committee [Shorrock, 1940]. When appetite was depressed so that the animals in any pen failed to eat the amount demanded by their live weight, they were fed according to appetite.

Once weekly, the pigs were given individually solutions containing their respective addenda. For this purpose, the pigs were placed one at a time in small wooden cages and offered the material mixed with a small quantity of meal. Owing to the large amount of zeaxanthin solution required by the three pigs receiving the higher level they were not separated but were given their zeaxanthin together during one feed each day.

Straw was used as litter in the first part of the experiment and wood shavings during the last few weeks.

Results

Preparation period. Some difficulty was experienced in the rearing and preparation of the experimental pigs. Two litters were reared with no more than normal losses during suckling. In one litter, however, six of the eight pigs alive after the first 27 days died during the suckling period. In most of these post-mortem examination showed inflammation of the intestines and pneumonia. The real cause of the losses was, nevertheless, obscure and there is little reason for attributing them solely to the diet at this early stage.

The vitamin A content of the livers of the animals which died during the preparation period is given in Table 2 (cf. p. 702). The data are few, but they show that the liver reserves at birth were small and they suggest that the amount was decreasing during suckling. The feeding of carotenoids or vitamin A was started when the pigs, according to litter, were 10 or 12 weeks old, and it is

Table 2. *Vitamin A in livers of pigs lost during the suckling and weaning stage*

	Litter A (Sow 185)	Litter B (Sow 576)				Litter C (Sow 607)			
No. born	12	17				11			
No. alive at beginning of experiment	10	11				8			
				Vitamin A in liver				Vitamin A in liver	
		Age at death days	Wt. liver g.	µg./g.	µg. in whole liver	Age at death days	Wt. liver g.	µg./g.	µg. in whole liver
Losses during preparation period	None	39	154	9·6	1478	18	123	10·8	1328
		74	74	7·8	577	27	223	9·1	2029
		80	151	7·8	1178	32	151	11·3	1706
						49	297	7·0	2079
						54	166	2·8	465
						57	247	4·3	1062
Remaining for grouping	10	8				2			

reasonable to assume that at this point the experimental animals had little or no vitamin A in their livers.

Experimental period

Growth. The growth curves of the twenty experimental pigs for the period of dosing are shown in Fig. 1.

All the pigs receiving vitamin A alcohol and vitamin A ester grew consistently throughout the experimental period. They grew at about the normal rate for pigs receiving good fattening rations and there was no evidence of any advantage of the higher level of vitamin A over the lower.

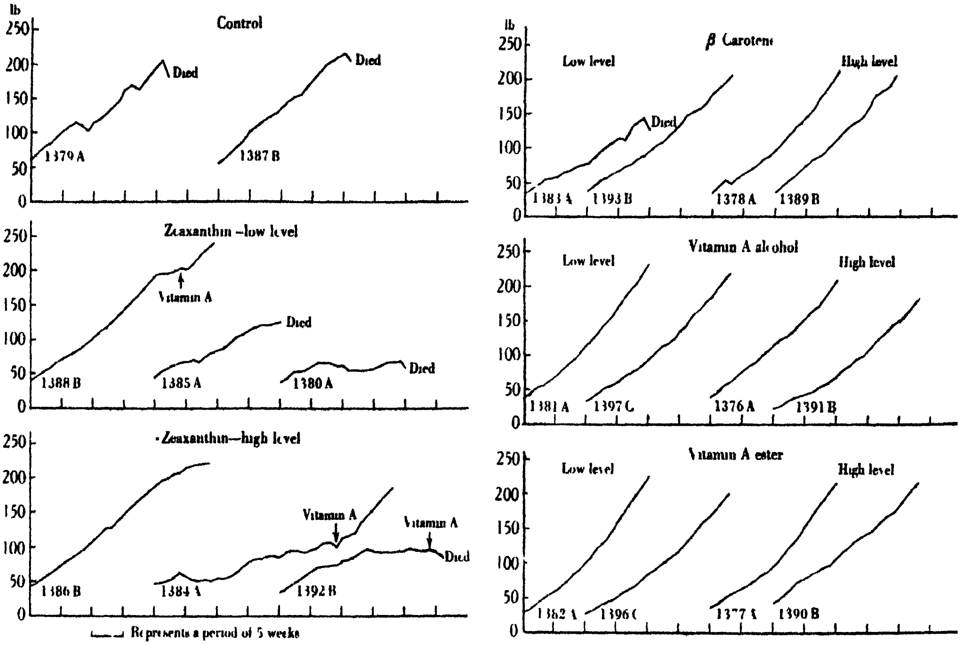


Fig. 1. Growth curves of experimental pigs

The two animals receiving the higher level of β-carotene also grew consistently and at a good rate. The growth of one on the lower level was, however, definitely subnormal and the animal eventually died 20 weeks after the commencement of the dosing.

The two control pigs were originally the biggest animals from two litters. They continued to grow, although in one case irregularly, for about 20 weeks. Growth then ceased and both pigs died a few days later.

There was much variation in the growth of the pigs receiving zeaxanthin. Two animals, one on the high level and one on the low level, grew normally for about 20 weeks. The first then became steady in weight and did not resume growth until given vitamin A, the second continued to grow at a very reduced rate. The remaining four pigs grew very little throughout the experiment; three of them died, two on the lower and one on the higher level; the fourth recovered after vitamin A treatment.

Health and symptoms. Of the twelve pigs which received vitamin A alcohol, vitamin A ester or β-carotene only one showed any symptoms or evidence of ill-health. This animal (no. 1383) was receiving the low level of β-carotene. Its appetite declined 15 weeks after the start of the experiment. It became thin, failed to thrive, developed laboured breathing and died 5 weeks later without showing any other symptoms.

The two negative control pigs showed no symptoms apart from occasional loss of appetite until the 20th week, when pig no. 1387 experienced a short convulsive fit from which it quickly recovered. The animal, however, became unstable on its legs and died 11 days later following two violent convulsive fits. The appetite of the other control pig, no. 1379, failed completely after the 21st week and the animal was unstable on its feet. Apart from that, however, no symptoms were shown and the pig died suddenly in the 22nd week.

The six zeaxanthin pigs all showed symptoms varying from only slight lack of appetite to typical convulsive fits. One animal, no. 1392, apparently became blind, while another, no. 1388, showed the typical irregular gait which we have often noted in previous experiments [Foot *et al.* 1938; 1939]. Hence the clinical evidence strongly suggested that the zeaxanthin at both the low and the high levels was biologically inactive.

Post-mortem findings. In the six pigs which died during the experiment, pneumonic changes, varying in intensity from slight congestion to grey hepatization, were the most conspicuous findings at necropsy. Patchy inflammation of the intestines, especially of the caecal region, was no less characteristic. This picture agrees with our earlier observations [Foot *et al.* 1938; 1939].

Curative treatment. Three pigs which failed to thrive when their diet was supplemented with zeaxanthin were given curative doses of vitamin A, 1500 I.U./10 lb. body weight daily. A spectacular cure was achieved in two cases (nos. 1384 and 1388); the third pig, which was obviously in a very poor state of health, failed to recover and died a few days after the curative attempt.

Vitamin A content of livers. The livers of all experimental pigs were examined for vitamin A by the method fully described by Foot *et al.* [1938]. The results are given in Table 3 expressed in micrograms of vitamin A. Values in brackets refer to animals which were given vitamin A as a curative measure. As a result of several estimations which agreed well together we have found that 1 Moore blue unit [Moore, 1930], as measured by us on vitamin A concentrates or on the dilutions used for pig feeding, is equivalent to 0·137 μg. vitamin A. The values are quoted on the assumption that the same factor would apply to the vitamin A present in pig livers. Dr A. E. Gillam kindly undertook to assay the livers by the spectrographic method. Unfortunately the results were with two exceptions rendered inconclusive because the vitamin peak was obscured by irrelevant absorption. Good vitamin A curves, however, were obtained for the livers of pigs nos. 1377 and 1388, especially for the former. From the extinction coefficients at 328 mμ it was calculated that these livers contained 22·3 and 139 mg. of vitamin A respectively. The corresponding figures calculated from the blue value measurements were 25·6 and 72 mg.

The complete absence of vitamin A from the livers of the three pigs which received only zeaxanthin is of particular interest and confirms the clinical evidence that this carotenoid is biologically inactive. The livers of the two pigs receiving the lower dose of β-carotene contained little or no vitamin A while the reserves of those given 300 I.U. of β-carotene per 10 lb. live weight were rather less than those of pigs given only 100 I.U. as vitamin A alcohol or ester.

Table 3. *Vitamin A in livers of experimental pigs*

Pig no. and litter	Age at death days	Wt. of liver g.	Vitamin A in liver	
			µg./g.	µg. in whole liver
Control 1379 A	249	1889	0	0
1387 B	229	2604	0	0
Zeaxanthin (low level)				
1380 A	233	923	0	0
1385 A	248	1505	0	0
1388 B	290	2009	(36·0)*	(72,324)*
Zeaxanthin (high level)				
1384 A	359	1501	(4·5)*	(6,754)*
1386 B	290	1500	0	0
1392 B	269	1057	(2·3)*	(2,431)*
Vitamin A ester (low level)				
1382 A	238	2094	1·3	2,722
1396 C	247	1698	1·3	2,207
Vitamin A ester (high level)				
1377 A	238	1684	15·2	25,597
1390 B	248	1698	8·8	14,942
β-Carotene (low level)				
1383 A	233	1796	0	0
1393 B	248	1698	Faint trace	Trace
β-Carotene (high level)				
1378 A	238	1953	0·5	976
1389 B	227	1698	0·3	509
Vitamin A alcohol (low level)				
1381 A	238	2066	1·4	2,892
1397 C	247	1868	0·9	1,681
Vitamin A alcohol (high level)				
1376 A	238	1670	9·1	15,197
1391 B	248	1613	7·8	12,581

* After receiving vitamin A.

Tests on rats

The substances given to pigs were also assayed on rats in curative tests. The technique was as described by Booth *et al.* [1934], with the minor modifications that dextrinized potato starch was used instead of dextrinized rice starch, that de Loureiro's [1931] salt mixture was used and that a small fraction of the yeast present in the diet was irradiated to supply vitamin D. The tests were of 4 weeks' duration. All test substances were given twice weekly, β-carotene, vitamin A alcohol and vitamin A ester at two levels of 2 and 4 I.U. daily and zeaxanthin at the presumptive levels of 4 and 12 I.U. (cf. p. 699). Groups of four male and five female rats were used for each substance. The results are given in Table 4.

Only three out of nine animals receiving the lower level of zeaxanthin survived the experimental period. There were two deaths at the higher level and the remaining seven rats lost weight heavily. The results show that zeaxanthin is of no value for the rat as provitamin A.

All rats receiving vitamin A alcohol, vitamin A ester and β-carotene survived, and gains in weight at corresponding levels of intake were very similar, indicating that all three substances had the same biological activity.

Table 4. *Weight gains of vitamin A-deficient rats receiving various supplements*

		Zeaxanthin					
4 I.U.				12 I.U.			
		4 weeks				4 weeks	
Rat no.	Initial wt. (g.)	Wt. (g.)	Gain (g.)	Rat no.	Initial wt. (g.)	Wt. (g.)	Gain (g.)
9877	110	Dead		9872	87	64	−23
9894	66	Dead		9901	85	65	−20
9902	78	Dead		9909	86	70	−16
9932	94	110	16	9933	90	97	7
9869	112	Dead		9876	94	Dead	
9881	63	Dead		9882	58	54	− 4
9905	76	Dead		9915	64	60	− 4
9922	136	108	−28	9926	116	90	−26
9929	98	85	−13	9935	71	Dead	
		Vitamin A alcohol					
2 I.U.				4 I.U.			
9885	51	66	15	9886	65	89	24
9910	94	106	12	9892	86	115	29
9919	81	93	12	9924	66	97	31
9939	66	82	16	9941	66	90	24
Mean ♂♂	73·0	86·8	13·8		70·8	97·8	27·0
9873	100	116	16	9874	73	84	11
9890	82	91	9	9898	64	76	12
9896	77	89	12	9906	74	87	13
9914	73	81	8	9928	96	105	9
9938	72	79	7	9937	65	80	15
Mean ♀♀	80·8	91·2	10·4		74·4	86·4	12·0
Mean ♂♂ and ♀♀	77·3	89·2	11·9		72·8	91·4	18·6
		β-Carotene					
2 I.U.				4 I.U.			
9879	101	112	11	9880	98	121	26
9903	101	114	13	9884	67	85	18
9908	82	99	17	9895	100	139	39
9918	73	90	17	9917	82	106	24
Mean ♂♂	89·3	103·8	14·5		86·7	113·5	26·8
9868	110	110	0	9870	92	110	18
9883	63	70	7	9904	81	100	19
9889	82	103	21	9913	66	88	22
9897	96	109	13	9921	116	125	9
9920	127	139	12	9936	78	78	0
Mean ♀♀	95·6	106·2	10·6		86·6	100·2	13·6
Mean ♂♂ and ♀♀	92·8	105·1	12·3		86·7	106·1	19·4
		Vitamin A ester					
2 I.U.				4 I.U.			
9887	66	74	8	9878	67	86	19
9893	74	86	12	9888	54	64	10
9900	70	88	18	9911	95	113	18
9934	107	131	24	9931	92	118	26
Mean ♂♂	79·3	94·8	15·5		77·0	95·3	18·3
9871	99	104	5	9867	111	121	10
9875	84	96	12	9899	80	105	25
9891	96	111	15	9912	70	96	26
9907	78	84	6	9923	85	106	21
9916	72	87	15	9930	108	113	5
Mean ♀♀	85·8	96·4	10·6		90·8	108·2	17·4
Mean ♂♂ and ♀♀	82·9	95·7	12·8		84·7	102·4	17·7

DISCUSSION

The results show without doubt that the pig, like the rat, is unable to make use of a hydroxy-β-ionone ring and cannot utilize zeaxanthin. Lund's [1938] results to the contrary were most probably due to active impurities present in his preparation of zeaxanthin. It is possible that the slight difference between the behaviours of the rats receiving in our experiments the two levels of this carotenoid may have been due to traces of carotene or cryptoxanthin retained in our preparation in spite of the precautions taken to secure a pure specimen.

A striking result of the present work is the confirmation of the findings of Guilbert *et al.* [1937] that the pig utilizes carotene less efficiently than vitamin A even under conditions probably not far removed from optimal. The two pigs receiving 100 I.U. of β-carotene per 10 lb. live weight were unable to store any vitamin A in their livers. One of them died and the post-mortem findings were typical of vitamin A deficiency. The two pigs receiving 300 I.U. of β-carotene grew satisfactorily, but their liver reserves were slightly lower than those of pigs given only one-third of this quantity as preformed vitamin A. Making due allowance for the small number of pigs used in our experiments we feel it safe to suggest that for the pig the efficiency of conversion of β-carotene into vitamin A is at best no greater than 30–40 %. On the other hand previously depleted rats utilized in our experiment β-carotene as efficiently as preformed vitamin A. The dosages were comparable with those received by the pigs, as 2 and 4 I.U. daily corresponded in this case to about 100 and 200 I.U./10 lb. live weight (Table 4). Though the treatment was preventive for pigs and curative for rats, both species were receiving similar levels of the provitamin dissolved in the same oil and the diets were also similar in being low in fat. It would seem therefore that the difference is a true one in metabolic behaviour.

The findings of Guilbert and his colleagues [Hart, 1940] point in the same direction and there are also indications that carnivora utilize carotene inefficiently as a source of vitamin A [Ahmad, 1931; Rea & Drummond, 1932; Frohring, 1935; cf., however, Bradfield & Smith, 1938]. Whatever the species, the ratio of the respective efficacies of vitamin A and carotene widens as the dose is increased [Guilbert *et al.* 1937; 1940]. Davies & Moore [1934] have reported that even the rat assimilates vitamin A at high levels much better than carotene. The careful tests of Wagner [1940] show the difference in the utilization of the provitamin and of vitamin A itself by man even after vitamin A deprivation. His findings are supported by numerous other reports, of which those of Booher *et al.* [1939], Booher & Calliston [1939] and Kreula & Virtanen [1939] may be quoted as examples. All these observations certainly bring out the complications and difficulties inherent in the use of carotene as a standard for vitamin A and make the suggestion of Guilbert *et al.* [1940] that separate standards for vitamin A and for carotene should be adopted worthy of serious consideration.

If the definition put forward by Guilbert *et al.* [1937] that the minimum level of vitamin A is one producing good growth but little storage in the liver is accepted, then our results are in good agreement with their findings. In our case the conditions were satisfied by 7·7 μg. of vitamin A as the alcohol or its natural esters and 40 μg. of β-carotene daily per kg. body weight. Figures in Table 1 show that the solutions of vitamin A alcohol and vitamin A ester deteriorated slightly during the tests. The loss of potency was of the order of 5 % and if taken into consideration would reduce the estimate of the minimum requirements to 7·3 μg. The figures given by the American workers for vitamin A and carotene were respectively 5·1–6·7 μg. and 25–39 μg.

The results of the rat assay of the vitamin A esters and vitamin A alcohol reported by us confirm the conclusions reached independently by Underhill [unpublished work] that there is no difference in the effect on the growth of vitamin A-deficient rats of equal quantities of vitamin A alcohol or natural vitamin A esters, when these quantities are estimated by measurement of the spectrographic absorption at 328 mμ. The significance of this result will be fully discussed by Underhill, so that it is only necessary to point out here that its attainment was probably due to the freedom of the preparations tested from oxidation products and other impurities which interfere with the spectrographic estimation.

The evidence as to the relative efficacies of free and esterified vitamin A for pigs fully supports the results obtained on rats, but owing to the use of fewer animals it carries less weight. There seems nevertheless to be some justification for the hypothesis that the two forms of the vitamin are equally, or nearly equally, effective for pigs.

SUMMARY

1. The vitamin A activities of carefully purified preparations of zeaxanthin, vitamin A alcohol, vitamin A ester and β-carotene were assayed on pigs and rats.

2. Zeaxanthin was biologically inactive for both species.

3. Pigs made less efficient use of β-carotene than rats and required, to cover their minimal requirements, about 300 I.U. of β-carotene or 100 I.U. of preformed vitamin A daily per 10 lb. live weight.

4. No difference was found in the biological activities of vitamin A alcohol and vitamin A natural ester for either pigs or rats.

We are greatly indebted to Dr N. S. Barron for carrying out the post-mortem examinations, to Dr E. Huntsman Mawson for the colorimetric tests on the zeaxanthin solutions and to Dr K. H. Coward for helpful criticism. One of us (T. H. M.) thanks the directors of the British Drug Houses, Ltd., for permission to publish that part of this work which was carried out in their laboratories.

REFERENCES

Ahmad (1931). *Biochem. J.* **25**, 1191.
Bacharach & Smith (1928). *Quart. J. Pharm.* **1**, 539.
Baxter & Robeson (1940). *Science*, **92**, 203.
Booher & Callison (1939). *J. Nutrit.* **18**, 459.
—— —— & Hewston (1939). *J. Nutrit.* **17**, 317.
Booth, Kon & Gillam (1934). *Biochem. J.* **28**, 2169.
Bradfield & Smith (1938). *Amer. J. Physiol.* **124**, 168.
Coward, Dyer & Morton (1932). *Biochem. J.* **26**, 1593.
—— —— —— & Gaddum (1931). *Biochem. J.* **25**, 1102.
Davies & Moore (1934). *Biochem. J.* **28**, 288.
Dunlop (1933). *J. agric. Sci.* **23**, 580.
—— (1934). *J. agric. Sci.* **24**, 435.
Edisbury, Gillam, Heilbron & Morton (1932). *Biochem. J.* **26**, 1164.
Embree (1939). *J. biol. Chem.* **128**, 187.
Emmet & Bird (1937). *J. biol. Chem.* **119**, xxxi.
Foot, Golding & Kon (1938). The vitamin requirements of pigs. Shinfield, Reading: National Institute for Research in Dairying.
—— Henry, Kon & Mackintosh (1939). *J. agric. Sci.* **29**, 142.
Frohring (1935). *Proc. Soc. exp. Biol., N.Y.*, **33**, 280.
Guilbert, Miller & Hughes (1937). *J. Nutrit.* **13**, 543.
—— Howell & Hart (1940). *J. Nutrit.* **19**, 91.
Hamano (1935). *Sci. Pap. Inst. phys. chem. Res., Tokyo*, **26**, 87.
Hart (1940). *Nutr. Abstr. Rev.* **10**, 261.
Hickman (1937). *Industr. Engng Chem.* (Industr. Ed.), **29**, 1107.
—— (1939). *J. biol. Chem.* **128**, xliii.

Holmes & Corbett (1937). *J. Amer. chem. Soc.* **59**, 2042.
Hume (1937). *Nature, Lond.*, **139**, 467.
—— (1939). *Nature, Lond.*, **143**, 22.
Kreula & Virtanen (1939). *Uppsala LäkFören. Förh.* **45**, 355.
Kuhn & Grundman (1934). *Ber. dtsch. chem. Ges.* **67**, 593.
de Loureiro (1931). *Arch. Patol., Lisboa,* **3**, 72.
Lund (1938). *Beretn. Forsögsla., Kbh.*, p. 180.
Mead, Underhill & Coward (1939). *Biochem. J.* **33**, 589.
Moll & Reid (1939). *Hoppe-Seyl. Z.* **260**, 9.
Moore (1930). *Biochem. J.* **24**, 692.
Morgan, Edisbury & Morton (1935). *Biochem. J.* **29**, 1645.
Morton (1940). *Analyst,* **65**, 263.
Petersen (1939). *Z. Schweinez.* **46**, 156.
Pritchard, Wilkinson, Edisbury & Morton (1937). *Biochem. J.* **31**, 258.
Rea & Drummond (1932). *Z. Vitaminforsch.* **1**, 177.
Reti (1935). *C.R. Soc. Biol., Paris,* **120**, 577.
Robinson (1938). *Biochem. J.* **32**, 807.
Shorrock (1940). *J. agric. Sci.* **30**, 598.
Strain (1938). Leaf xanthophylls. Washington: Carnegie Institution.
Tischer (1938). *J. biol. Chem.* **125**, 475.
Wagner (1940). *Hoppe-Seyl. Z.* **264**, 153.

74. METABOLISM OF STEROIDS

2. THE ISOLATION OF CHOLESTANE-3:5:6-TRIOL AND OTHER SUBSTANCES FROM OX LIVER EXTRACTS

By G. A. D. HASLEWOOD

From Guy's Hospital Medical School, Sherwood Park, Tunbridge Wells

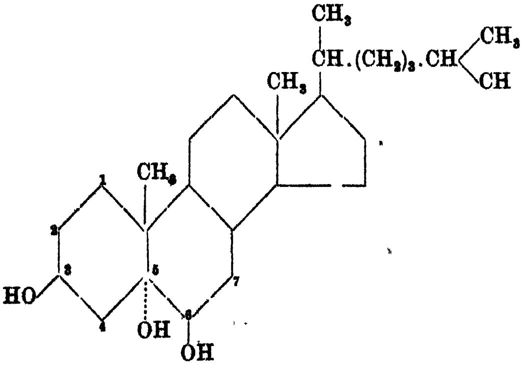
(*Received 14 May 1941*)

In Part 1 [Haslewood, 1939], the isolation from ox liver of α-7-hydroxycholesterol was reported. Since that time, a 'β-7-hydroxycholesterol' has been mentioned as isolated from hog liver [MacPhillamy, 1940], and β-7-hydroxycholesterol has been obtained from pregnant mares' serum [Wintersteiner & Ritzmann, 1940]. Wintersteiner & Bergström [1941] report further that cholesterol can be converted into a mixture containing 7-hydroxy- and 7-keto-cholesterol by treatment at an alkaline reaction with oxygen at 85°. As Wintersteiner and his colleagues ably and correctly point out, all this work does not answer the question of the authenticity of the oxygenated cholesterols as metabolites, since it would seem possible that such oxidation products are formed from cholesterol during the processes leading to their isolation. Oxidation of cholesterol appears to take place readily at the double bond, as well as at position C_7, as would be expected on chemical grounds, but whether the products are actually those taking part in metabolism can only be decided by many types of experiment, including, perhaps, such work as the treatment of cholesterol with enzymic preparations.

The present work was carried out on the same fraction of ox liver as was previously used, and from this source there has been obtained an incompletely purified *alcohol*, probably $C_{24}H_{46}O_3$, characterized as the *monoacetyl derivative*, $C_{24}H_{45}O_2(O.CO.CH_3)$. This substance is not of the steroid type.

α-7-Hydroxycholesterol (identified as the dibenzoate) has been obtained from the extract directly as crystals melting at 174–176°: the compound is difficult to crystallize [cf. Wintersteiner & Ritzmann, 1940].

There has also been isolated from the liver extract cholestane-3:5:6-triol, identified as the diacetate. This triol is obtained by oxidation of cholesterol or its acetate with H_2O_2 [Pickard & Yates, 1908] and has been called 'triol I' by Ellis & Petrow [1939], who assign to it the structure:



Many authors have stressed the analogy between biological oxidations and those brought about by H_2O_2; nevertheless the foregoing remarks about the origin of substances isolated apply to cholestane triol, especially since ether was used in its preparation from liver.

As already reported [MacPhillamy, 1940; Haslewood, 1941], the 'hepatols' have been derived from digitonin used in their preparation. One of them, 'hepatol A', is apparently digitogenin, $C_{27}H_{44}O_5$, as stated by MacPhillamy [1940]. Its confusion with a C_{21} compound arose because of the difficulty of obtaining an accurate measurement of its molecular weight. The problem was solved when the diacetate was oxidized with cold chromium trioxide to a monocarboxylic acid, probably $C_{30}H_{45}O_7.COOH$, which was titrated. This acid was obtained in good yield; its formation is explicable in terms of the formula assigned to digitogenin by Marker & Rohrmann [1939], although it is difficult to understand why digitogenin (with hydroxyl groups at C_2, C_3 and C_6) should not easily be completely acetylated.

The cholestane triol was first obtained as the digitonide. This was decomposed with pyridine and acetic anhydride and from the acetylated mixture the diacetate of the triol was readily crystallized.

EXPERIMENTAL

Analyses were microanalyses by Dr A. Schoeller (S) and Dr G. Weiler (W). All melting points are uncorrected.

Starting material was the evaporated methyl-alcoholic mother liquors (fraction A) from the crystallization of the 'sterols' from the unsaponifiable ether-soluble fraction of ox-liver 'marc' [Haslewood, 1941].

The alcohol (?) $C_{24}H_{46}O_3$. Fraction A (20 g.), on standing in solution in about 20 ml. of ether, or ether in light petroleum, slowly deposited a semi-crystalline solid (0·1 g.) which was collected and crystallized from acetone. It formed colourless globules of small crystals, M.P. 93–95°, not soluble in alkali, readily forming a 'gel' from dilute alcohol and giving negative tests for N and P. The substance gave no precipitate with digitonin or with 2:4-dinitrophenylhydrazine in dilute alcoholic solution. Found (W): C, 74·85, H, 12·4 %, mol. wt. = 353. $C_{24}H_{46}O_3$ requires C, 75·4, H, 12·0 %, mol. wt. = 382.

This material (50 mg.) in pyridine (0·2 ml.) with acetic anhydride (0·2 ml.) was heated at 100° for 15 min. The precipitate collected after dilution with water was recrystallized from acetone and separated as clumps of white needles, M.P. 103–105°. Found (W): C, 73·8, H, 11·3 %, mol. wt. = 428. $C_{24}H_{45}O_2(O.CO.CH_3)$ requires C, 73·6, H, 11·3 %, mol. wt. = 424. A sample from the mother liquors had M.P. 107–108°, while another obtained after six crystallizations from acetone of the 'sterols', acetylated with acetic anhydride, melted at 100–111°, both samples being saponified with dilute alcoholic NaOH to the above alcohol. These facts suggest that the material has not been completely purified, though all samples of the acetate had sharp melting points.

Separation of hydroxylated cholesterols. Fraction A (16 g.) was dissolved by shaking with 100 ml. of 90 % (by vol.) aqueous methyl alcohol and 100 ml. of light petroleum (B.P. 40–60°). The alcoholic layer was separated and washed in a funnel with two portions each of 50 ml. of fresh petroleum. The combined petroleum on evaporation gave 14·3 g. of *fraction B*, while evaporation of the alcohol yielded *fraction C* (1·7 g. of an orange gum). Fraction C was dissolved in 25 % (by vol.) benzene in light petroleum and allowed to run through a cylindrical column (length 25 cm., diam. 0·8 cm.) of Al_2O_3 (Hopkin & Williams).

The column was then washed with solvents as follows, the eluates being collected separately and evaporated, and the weights of the residues being obtained. In this way, fractions were collected as below:

Fraction	Solvent	Weight g.	Description of residue
I	Up to 100 ml. of 25% benzene in light petroleum	0·2	Mobile oil
II	100 ml. of benzene	0·15	,,
III	100 ml. of ether	0·8	Stiff gum
IV	100 ml. of warm alcohol	0·2	,,
V	100 ml. of alcohol acidified with HCl and warmed with the disintegrated column; product isolated with ether, after dilution	0·17	,,

Fraction III (0·8 g.) in benzene was adsorbed on a fresh column (20 × 0·8 cm.) and the column eluted with 100 ml. of 50% (by vol.) benzene in ether (residue (0·55 g.): fraction III*a*) and then with 100 ml. of alcohol (residue (0·25 g.): fraction III*b*).

Fraction III a (0·55 g.) in benzene was adsorbed on a fresh column (10 × 0·8 cm.) and was eluted with benzene up to 500 ml. Evaporation of the solvent gave solid α-7-hydroxycholesterol, which, from benzene in light petroleum, formed long white needles, M.P. 174–176° decomp. Yield, ca. 10 mg. 5 mg. of this product on benzoylation with PhCOCl (1 drop) and pyridine (2 drops) for 2 hr. at room temperature gave on dilution a gum which was crystallized from methyl alcohol to give white needles of α-7-hydroxycholesterol dibenzoate, M.P. 170–171°, not depressed by authentic material, M.P. 170–172°. The hydroxycholesterol and its dibenzoate gave the characteristic colour and precipitation reactions.

Fraction III b (0·25 g.) was warmed with a solution of digitonin (0·2 g.) in 20 ml. of 80% alcohol. A crystalline digitonide separated rather slowly and after 24 hr. was collected, washed with alcohol and ether and dried. The product (50 mg.) in pyridine (0·5 ml.) with acetic anhydride (0·5 ml.) was heated for 20 min. at 100°. The precipitate obtained after dilution with water was collected and dissolved in warm alcohol. When the warm filtered solution was diluted to faint cloudiness and allowed to stand, needles separated and were collected and recrystallized from dilute alcohol, from which separated long glistening needles (8 mg.), M.P. 163–165°, not depressed by cholestane triol I diacetate, M.P. 167–168°, prepared by acetylation by the above method of the purified triol. Found (W): C, 73·4, H, 10·6%, M=453, 480. $C_{31}H_{52}O_5$ requires C, 73·8, H, 10·3%, mol. wt. = 504. Saponification gave cholestane triol (M.P. 235–237°) identical with authentic material. 40 mg. of authentic triol (M.P. 233–235°), obtained from cholesterol with H_2O_2, together with 0·1 g. of digitonin, were dissolved by warming with 15 ml. of 80% alcohol. After standing for 24 hr., the crystalline digitonide (80 mg.) was collected, washed, dried and acetylated as above. The acetate obtained had M.P. 163–165°, not depressed by the specimen from liver.

'Hepatol A' from digitonin. Digitonin (0·2 g.), with a few drops of a dilute solution of Br_2 in alcohol, was refluxed for 2 hr. with 2 ml. of xylene. The xylene was evaporated and the residue purified by washing with light petroleum, sublimation and crystallization as previously described. White needles, M.P. 275–278°, not depressed by 'hepatol A', were obtained.

The acid $C_{30}H_{45}O_7.COOH$. The diacetate (10 mg. of M.P. 235–238°, and prepared as previously described) of the above compound was dissolved in glacial acetic acid (0·2 ml.) and the mixture allowed to stand at room temperature for 16 hr. with 0·2 ml. of a solution made by dissolving 10 g. of CrO_3 in the minimal amount of water and making up to 100 ml. with acetic acid. After dilution with water, the precipitate was collected and recrystallized from dilute alcohol, from which it formed white needles, M.P. 263–264°, decomp. Yield, 7 mg. Found (S): C, 65·7, H, 8·2 %, mol. wt. (titration) = 540, 519. $C_{30}H_{45}O_7.COOH$ (one carboxyl group) requires C, 66·2, H, 8·2 %, mol. wt. = 562. The above compound was easily soluble in warm dilute $NaHCO_3$ solution, from which it was precipitated on acidification. It showed no alteration after treatment with acetic anhydride and pyridine and gave no obvious reactions with ketone-detecting substances. With diazomethane, a partially purified methyl ester, M.P. 184–186°, was formed. Found (S): C, 66·3, H, 8·5 %. $C_{30}H_{45}O_7.COOCH_3$ requires C, 66·7, H, 8·3 %.

SUMMARY

In addition to crystalline α-7-hydroxycholesterol, M.P. 174–176°, there has been obtained from the unsaponifiable fraction of an ether extract of ox liver cholestane-3:5:6-triol I, and an alcohol, M.P. ca. 95°, characterized as a monoacetyl derivative. 'Hepatol A' is digitogenin: its diacetate has been oxidized to a monocarboxylic acid.

Experiments of the type now reported do not finally establish the authenticity of isolated substances as intermediates in metabolism.

The author's sincere thanks are due to Messrs Boots Pure Drug Co. for extracting and saponifying a large quantity of ox liver marc, and also to Prof. R. A. Peters for generous hospitality accorded at the Biochemical Laboratories, Oxford.

REFERENCES

Ellis & Petrow (1939). *J. chem. Soc.* p. 1078.
Haslewood (1939). *Biochem. J.* **33**, 709.
—— (1941). *Chem. & Ind:* **60**, 220.
MacPhillamy (1940). *J. Amer. chem. Soc.* **62**, 3518.
Marker & Rohrmann (1939). *J. Amer. chem. Soc.* **61**, 2724.
Pickard & Yates (1908). *J. chem. Soc.* **93**, 1678.
Wintersteiner & Ritzmann (1940). *J. biol. Chem.* **136**, 697.
—— & Bergström (1941). *J. biol. Chem.* **137**, 785.

75. THE STEROL REQUIREMENTS OF THE LARVA OF THE BEETLE, *DERMESTES VULPINUS* FABR.

BY G. FRAENKEL, J. A. REID AND M. BLEWETT

From the Department of Zoology and Applied Entomology, Imperial College, London

(*Received 27 May 1941*)

HOBSON [1935] showed that blowfly larvae required a fat-soluble growth factor which was contained in the unsaponifiable ether-soluble fraction of their food and proved to be identical with or replaceable by cholesterol. Subsequently the indispensability of cholesterol for the growth of many insects was demonstrated (Van t'Hoog [1936] for *Drosophila*; Chiu & McCay [1939] for the bean weevil *Acanthoscelides obtectus*; Fröbrich [1939] and Offhaus [1939] for the flour beetle *Tribolium confusum*; and for the edible snail, *Helix pomatia*, Howes [1937]). In a study of the nutritional requirements of the beetle *Dermestes vulpinus*, carried out in this department, Gay [1938] showed that growth of the larva was satisfactory on a diet consisting of casein, cystine, yeast, salt mixture and cholesterol, or of yeast, salts and cholesterol, while omission of cholesterol from either diet prevented growth. It has been the purpose of the present investigation to obtain more detailed information about the sterol requirements of this beetle.

EXPERIMENTAL

The technique of breeding was very similar to that employed by Gay [1938]. The method of obtaining eggs and newly hatched larvae has been described by Dick [1937]. The insects were bred at 27° and 70 % relative humidity except in the last series of experiments (Tables 5–7), when the temperature was 25°. It was at first thought that the larvae would attack each other and to prevent such loss of animals by cannibalism they were bred singly in glass tubes, the history of growth of each larva being followed, as a rule by daily inspection, until pupation or death. This precaution, however, later proved unnecessary and in the last series five larvae were reared together in a tube.

The following diets were used:

Diet I (called subsequently 'casein diet'): 3·0 g. ashless vitamin-free casein (casein E, Glaxo Laboratories), 0·3 or 0·15 g. dried debittered brewer's yeast (Glaxo Laboratories), 0·1 g. sterol, 0·02 g. cystine (B.D.H.), 0·03 g. MacCollum's salt mixture, distilled water to 50 % by weight.

Diet II (called subsequently 'yeast diet'): 3·0 g. dried debittered brewer's yeast (Glaxo Laboratories), 0·1 g. sterol, 0·03 g. MacCollum's salt mixture, distilled water to 50 % by weight.

Diet III (described on p. 713).

The following sterols were tested, their source being indicated in the list:

From Glaxo Laboratories: cholesterol, sitosterol, ergosterol, calciferol, 7-ketocholesteryl acetate, 7-hydroxycholesterol, 7-hydroxycholesteryl dibenzoate, 7-dehydrocholesteryl monobenzoate, 7-dehydrocholesterol.

From Prof. I. M. Heilbron and Dr E. R. H. Jones: cholesterol purified via the dibromide, cholestanol (dihydrocholesterol), zymosterol, Δ^5-cholestene, cholesteryl acetate.

At a temperature of 27 or 25°, a humidity of 70 % and a water content of 50 % the diet would be expected to become infested with moulds and other micro-organisms within a few days. This might seriously alter the composition of the food, apart from introducing new food factors supplied by the micro-organism. Serious efforts were made to limit the degree of infestation and four different methods were tried for this purpose.

1. An attempt was made to grow the larvae under sterile conditions. The eggs were sterilized by immersion in a 3 % solution of $HgCl_2$ for 3 min. The food was sterilized by heating the dry constituents, minus the yeast, at 145° for 1½ hr., and to them was added a suspension of yeast in water which had been brought to the boil. Two days later this mixture was again heated at 100° for 10 min. While the cultures seemed to remain sterile for about 12–16 days, almost invariably moulds then began to grow, most probably due to infestation from outside. Although it seemed likely that lasting sterility could be achieved by further improvement of the technique the drawbacks of the method were so manifold that it was finally abandoned.

2. During the past few years increasing use has been made of the antiseptic properties of the esters of *p*-hydroxybenzoic acid for keeping organic media free from bacteria and moulds [e.g. Cremer, 1935]. The methyl ester, known under the name 'Nipagin M', is used in this connexion in the breeding of *Drosophila*. We found in a series of experiments that admixture of 0·5 % of Nipagin in the wet casein diet prevented growth of moulds in a varying proportion of the cultures while having no influence on the growing larvae. On the other hand, moulds never appeared in a yeast diet to which 0·5 % Nipagin had been added. In spite of the uncertain effect of Nipagin as a fungicide, useful results could be obtained with this method.

3. The food was changed daily to prevent the development of moulds. This method was previously used by Gay [1938] and was adopted for the first of the present series of experiments. Although it yields very reliable results it has the disadvantage of being very laborious and requiring large amounts of the diets.

4. After many of the experiments had been completed the following diet was devised, based on the strong hygroscopic properties of fructose:

Diet III (called subsequently 'yeast fructose diet'): 1·5 g. dried debittered brewer's yeast (Glaxo Laboratories), 1·5 g. fructose, 0·05 g. sterol (or less), 0·03 g. MacCollum's salt mixture (not essential and usually omitted), 0·4 ml. water. This quantity was enough for five larvae, which were reared together in one tube. Owing to the hygroscopic properties of fructose the diet, under the conditions at which it was used (25° and 70 % relative humidity), acquired a water content of 20 to 25 %, and the high sugar content prevented growth of moulds very effectively. One of the minor disadvantages of the diet was that the water content was not exactly the same in different samples, and since in *Dermestes* the rate of growth is very much dependent on the water content of the diet [Gay, 1938] any minor differences which were recorded in the duration of larval life could not be regarded as significant. An attempt to breed *Dermestes* on a casein diet to which 50 % fructose was added proved less successful because the diet got so wet that many young larvae were drowned in it.

In all experiments except the later ones on the fructose diet, the development of each individual larva was observed up to the time of pupation, and the subsequent emerging of the adult and the number and time of each moult were recorded. The data presented as a measure of the efficiency of a diet are limited in this paper for the most part to the times until the larvae pupated or, in case of an unsuccessful diet, died. The duration of larval life is the only figure of significance, since growth takes place only during that period. Once a larva has pupated, the duration of the pupal stage is constant (about 7 days at 27°). The adults were always fertile. The controls were reared on fishmeal which was moistened to contain 50 % water.

The first question to be decided was whether the positive effect of cholesterol on growth was not due to the presence of impurities such as 7-dehydrocholesterol, the natural provitamin D_3 which is considered to be present in commercial cholesterol. Series 1 compares the effect of commercial cholesterol (B.D.H.) with a sample of cholesterol specially purified (via the dibromide), which was free of 7-dehydrocholesterol. Although for reasons not connected with the course of the experiment this series had to be abandoned after 11 days, it was by then clear that the larvae grown on the two samples of cholesterol hardly differed with regard to mortality and speed of development and were slightly more advanced in development than the controls (Table 1). In the absence of cholesterol, development was slow, the mortality was high and the few surviving larvae were in an obviously dying condition.

Table 1

No.	Diet	Condition after 11 days	
1	Fishmeal (control)	9 in 4th instar	1 dead
2	Yeast diet, commercial cholesterol	9 in 5th instar	1 dead
3	Yeast diet, purified cholesterol ✓	6 in 5th instar 4 in 4th instar	0 dead
4	Yeast diet, no sterol	2 in 3rd instar 1 in 4th instar	7 dead

Temp. 27°. Food changed daily.

Series II (Table 2) was devised to compare the effect of purified cholesterol with those of sitosterol, ergosterol and calciferol. The first three sterols were incorporated in a casein diet containing 5 % yeast and the food was changed daily. This method was not used with calciferol, owing to the expense involved; the Nipagin method was used instead.

It can be seen in Table 2, nos. 7–9, that the diets containing ergosterol or calciferol were entirely inadequate. In fact the larvae died as rapidly as if no sterol had been present [cf. Gay, 1938]. Sitosterol proved of slight nutritional

Table 2

No.	Diet	Method	No. of larvae	No. pupating	Mean time to pupation days
5	Pure, cholesterol, casein diet, 5 % yeast	Food changed daily	10	5	39·8
					Mean time to death days
6	Sitosterol, casein, diet, 5 % yeast	Food changed daily	10	Last larva died after 30 days (one pupated 47th day)	18·2
7	Ergosterol, casein diet, 5 % yeast	Food changed daily	10	Nil. Last died after 13 days	10·4
8	Calciferol, casein diet, 5 % yeast	0·5 % Nipagin; 3 tubes grew mould 11th day	15	Nil. Last died after 15 days	12·3
9	Calciferol, yeast diet	0·5 % Nipagin; no mould	15	Nil. Last died after 14 days	10·1

Temperature 27°.

value. Only one larva reached the pupal stage after long delay and the remaining larvae lived considerably longer than on ergosterol and calciferol. The course of the experiment on pure cholesterol was not entirely satisfactory. The mortality was somewhat higher than it should have been and the time to pupation of 39·8 days compares unfavourably with Gay's figure of 29·2 days, which was obtained, however, in experiments with commercial cholesterol.

Series III (Table 3) was devised to compare the efficiencies of commercial and pure cholesterol. To make the general conditions more favourable a casein diet with 10 % (instead of 5 %) of yeast was used. It was desirable to know whether any fat-soluble factors in yeast other than sterols would play a role in the nutrition of *Dermestes*. The effect of ether-extracted yeast (extracted for 7 days at room temperature, ether changed once or twice daily) was therefore compared with that of ordinary yeast. The series was set up with the Nipagin method, and in order to obviate possible water loss from the diet it was changed once on the 15th day. In order to make the conditions strictly comparable for all the sterols used further tests with ergosterol and sitosterol were included.

Table 3. *Series III. Casein diet with 10 % yeast. 0·6 % Nipagin added; food changed once on the 15th day*

No.	Diet	No. of larvae	No. pupating	Mean time to pupation, days
10	Commercial cholesterol	15	11	26·0
11	Pure cholesterol	15	11	26·0
12	Commercial cholesterol ether-extracted yeast	15	11	29·2
13	Pure cholesterol ether-extracted yeast	15	14	28·6
			Last died after, days	Mean time to death, days
14	Ergosterol	15	31	15·8
15	Sitosterol	15	34	20·0

Temperature 27°.

Comparing nos. 10 and 11 and nos. 12 and 13 it is obvious that there is no difference in the effects of commercial and of pure cholesterol. It is therefore clear that the effective substance for the growth of *Dermestes* is cholesterol and not 7-dehydrocholesterol. It is not likely that any ether-soluble constituents of yeast are required. The slight prolongation of larval life on a diet containing ether-extracted yeast is not considered to be significant and may well be due to traces of the ether remaining in the yeast. Ergosterol and sitosterol again had no effect. Growth was very slow and all larvae died long before reaching the state of pupation, although single larvae remained alive for a considerable period. The longer survival on sitosterol and ergosterol and the quicker rate of development on cholesterol seen in Table 3, compared with those seen in Table 2, are almost certainly due to the larger amount of yeast in the diet.

In the experiments in which the food was mixed with Nipagin and was not changed daily some growth of mould usually occurred. In some tests only a small proportion of the tubes developed mould, in others a large proportion, though in none was the growth strong and it was always delayed in appearance. The data given in Table 4 show that the slight growth of mould that occurred had no significant effect upon the results of the experiments. In most instances the mean time until death or pupation of all larvae was not significantly different

Table 4. *Casein diets* (*except no. 9*)

No.	Medium	No. of tubes	No. with mould	Mean time to appearance of mould days	Mean time to death or pupation of all larvae days	Mean time to death or pupation of those exposed to mould days	
9	Calciferol, yeast diet, 0·5 % Nipagin	15	0	—	10·1	—	Time to death
8	Calciferol, 5 % yeast, 0·5 % Nipagin	15	3	10·0	12·3	12·0	
15	Sitosterol, 10 % yeast, 0·6 % Nipagin	15	5	12·0	20·0	17·4	
14	Ergosterol, 10 % yeast, 0·6 % Nipagin	15	2	19·0	15·8	20·0	
11	Specially purified cholesterol, 10 % yeast, 0·6 % Nipagin	15	13	11·0	26·0	25·8	Time to pupation
10	Commercial cholesterol, 10 % yeast, 0·6 % Nipagin	15	10	9·0	26·0	25·8	
13	Specially purified cholesterol, 10 % ether-extracted yeast, 0·6 % Nipagin	15	5	16·6	28·6	29·2	
12	Commercial cholesterol, 10 % ether-extracted yeast, 0·6 % Nipagin	15	9	10·8	29·2	31·8	

from the mean times of those alone in which mould occurred. In only one case (no. 15) did the mould seem to have an adverse effect and in only one case (no. 14) a beneficial effect.

Long after the experiments so far described had been concluded the new yeast fructose technique, described on p. 713, was devised and new series of experiments were set up (Tables 5 and 6). In view of the large amounts of yeast present (50 % of dry diet) it was not considered essential to add salts. In fact, addition of salt mixture did not alter the result if cholesterol or any other growth-promoting sterol was present. When, however, no sterols were supplied, the addition of salts usually had the effect of delaying death in a few individuals. As a consequence no salts were added in the experiments of Table 6 except where expressly stated (no. 21).

It is obvious from Table 5, nos. 16 and 17, that the activities of pure cholesterol and 7-dehydrocholesterol are about equal, although smaller quantities of the latter were used. The periods required for pupation in this series are considerably longer than in those of former experiments (Table 3). This is due mainly to the lower temperature of breeding (25 against 27°) and the lower water content (20–25 against 50 %); it may also have been due in part to the lower protein content of the fructose diet and the breeding of five larvae together in one tube, instead of single cultures. On the other hand, the lower death rate in the fructose diet experiments suggests that the conditions must have been more favourable in other respects.

Of thirteen sterol preparations tested (Tables 5 and 6) only four proved to be of any nutritional value: cholesterol, dehydrocholesterol and two esters of these substances, cholesteryl acetate and 7-dehydrocholesteryl monobenzoate.

Table 5. *Yeast fructose diet; 50 mg. of sterol (10 mg. in case of dehydrocholesterol) in 3 g. of food; 25°.*

No.	Sterol	No. of larvae	No. of pupae	First pupa at day	Last pupa at day	Average time of pupation days
16	Cholesterol	10	10	33	39	36
17	7-Dehydrocholesterol, 10 mg.	10	10	33	38	35·5
18	Cholesteryl acetate	10	10	32	41	36·5
19	7-Dehydrocholesteryl monobenzoate	10	8	33	39	36

Table 6. *Yeast fructose diet; 50 mg. of sterol in 3 g. of food; 25°*

No.	Additions to diet	No. of larvae	First dead at day	Last dead at day	Average time of death days
20	No sterol, no salts added	30	16	22	19
21	No sterols, salts added	10	16	53*	24
22	Sitosterol	20	18	57*	23·4
23	Ergosterol	20	16	25	20·5
24	Zymosterol	20	16	18	16·5
25	Chloroform extract of wholemeal flour	20	16	21	18·5
26	Cholestanol	20	16	21	18·5
27	Cholestene	10	17	37* (1 pupa 53 days)	21
28	7-Ketocholesteryl acetate	20	16	48*	19·8
29	7-Hydroxycholesterol	20	16	40*	18·8
30	7-Hydroxycholesteryl dibenzoate	20	16	40* (1 pupa 42 days)	19

* One larva only survived for any considerable time.

The differences in the average survival times are not considered to indicate significant differences in the potencies of the inadequate diets. It was not considered advisable to disturb cultures too often, and at the first inspection usually several larvae were dead, so that the figures of the last column cannot be regarded as accurate mean values. The fact that on several diets one larva survived the others for a considerable time, and on cholestene and 7-hydroxycholesteryl dibenzoate even reached pupation, may be of some significance, but is more likely due to the presence of impurities in the sterols. This is the more probable since the chloroform-soluble fraction of whole wheat, which besides sitosterol certainly contained mixtures of other sterols and which proved to be an adequate source of sterols for several flour insects (unpublished), was entirely negative.

This also raised the question of whether some of the positive effects of sterols (Table 5) may not equally be due to impurities. The preparations which were available, with the exception of cholesterol, were admittedly not chemically pure, and it was possible that the presence of cholesterol in 7-dehydrocholesterol and in 7-dehydrocholesteryl monobenzoate might be the cause of their being of positive nutritional value. Chemically pure samples of these compounds not being obtainable, an attempt was made to determine the lowest effective concentration of each of these compounds and of pure cholesterol. Only if this concentration proved to be considerably lower in the case of cholesterol than in that of the other compounds could the presence of small quantities of cholesterol as an impurity possibly give them an apparent positive nutritional value. It was also considered of interest to ascertain whether the presence of such large quantities of sterols as had hitherto been used was essential or whether the quantities required might be more of the order of vitamin requirements.

Accordingly, series of diets with decreasing quantities of sterols were set up (Table 7). The threshold of 7-dehydrocholesterol was found to be considerably lower than that of cholesterol. Decreasing the quantities of 7-dehydrocholesterol from 10 to 0·5 mg./3 g. of food hardly altered the efficiency of the diet, so that the threshold must be considerably lower than this. With cholesterol at quantities below 2 mg. the efficiency decreased considerably, as seen by an increasing mortality and increasing time to pupation. Since, therefore, 7-dehydrocholesterol is more effective at low concentrations than cholesterol, the possibility that cholesterol as an impurity is the cause of the positive effect given by 7-dehydrocholesterol is ruled out. The same consideration applies to 7-dehydrocholesteryl monobenzoate, the threshold of action of which does not differ greatly from that of cholesterol.

Table 7. *Yeast fructose diet; 25°*

No.	Additions to diet mg./3 g. food	No. of larvae	No. pupating	First pupa at day	Last pupa at day	Average time of pupation days
31 (=16)	Cholesterol, 50	10	10	33	39	36
32	„ 10	10	10	31	41	36·5
		10	10	35	42	36·7
33	„ 2	10	10	33	41	37·9
		10	10	35	41	37·3
34	„ 1	10	6	35	45	40
35	„ 0·5	10	2	39	49	44
36 (=17)	Dehydrocholesterol, 10	10	10	33	38	35·5
37	„ 2	10	10	33	42	37·5
38	„ 1	10	9	33	41	37
39	„ 0·5	10	9	33	41	37
40	Dehydrocholesteryl monobenzoate, 5	10	10	34	45	38
41	„ 2	10	10	38	46	41
42	„ 1	10	9	38	57	47·8
43	„ 0·5	10	4	45	57	50·2

The experiments of Table 7 are, of course, not real threshold experiments, because the quantity of the substance which the insects have actually eaten during development is unknown. The amount of dehydrocholesterol in the food was not further decreased, since it was not believed possible to mix such small amounts evenly into the food.

Discussion

The only previous attempt to compare the roles of several sterols as food factors was undertaken by Van t'Hoog [1936] on *Drosophila*. *Drosophila* larvae were found to grow well on a sterile diet consisting of peptone from purified casein, sugar, a collodion dialysate of yeast extract, salts, agar-agar and water, if to this food was added a small quantity of the unsaponifiable fraction of fat [Van t'Hoog, 1935]. Later, Van t'Hoog [1936] found that this unsaponifiable fraction could be replaced successfully by a number of sterols: cholesterol (purified via the dibromide), sitosterol, stigmasterol, ergosterol, cholestanol (dihydrocholesterol) and cholestenone. Calciferol, on the contrary, and several derivatives of cholesterol, proved to be ineffective. The flour beetle *Tribolium confusum* also requires in its diet either cholesterol or ergosterol [Fröbrich, 1939; Offhaus, 1939]. The differences in the sterol requirements of two different insects, *Drosophila* and *Dermestes*, are remarkable. Cholesterol, sitosterol and stigmasterol possess the same ring system with only one double bond in ring B and differ solely in the side chain on carbon atom 17. This difference is of no importance for *Drosophila* and of great importance for *Dermestes*, which cannot develop on sitosterol. Ergosterol and 7-dehydrocholesterol contain a second double bond in ring B and differ from each other only in the side chain. While ergosterol is active for *Drosophila* and *Tribolium* it is entirely inactive for *Dermestes*. This negative effect on *Dermestes* is obviously due to the side chain and not to the second double bond, because 7-dehydrocholesterol, which possesses the ring system of ergosterol and the side chain of cholesterol, is active for *Dermestes*. Cholestanol, which differs from cholesterol only in the absence of the double bond in ring B, is positive for *Drosophila* and negative for *Dermestes*, which indicates that the presence and position of the double bond is of importance too. Zymosterol, with one double bond in a different place in ring C and a side chain which is different from that of cholesterol, is also inactive.

Of the derivatives of cholesterol and dehydrocholesterol tested, only the esters of these compounds, cholesteryl acetate and 7-dehydrocholesteryl monobenzoate, have a positive effect, which suggests that hydrolysis of these esters takes place in the larva. Other changes in the configuration, such as the loss of the double bond in cholestanol, loss of the secondary alcohol group in Δ^5-cholestene, introduction of a second alcohol group in 7-hydroxycholesterol and esterification of this group in 7-hydroxycholesteryl dibenzoate or introduction of a keto-group on carbon atom 7 in 7-ketocholesteryl acetate, cause loss of nutritional value. It therefore appears, as far as can be judged from the number of compounds available for testing, that only substances with the configuration of cholesterol and dehydrocholesterol or their esters can be utilized by *Dermestes*. Change in the side chain, the number and position of double bonds, opening out of one ring, introduction of a second alcohol or a keto-group destroy the effect entirely.

The differences in the requirements for sterols in *Drosophila* and *Dermestes* are interesting. *Dermestes* feeds exclusively on relatively dry products of animal origin like stored hides, bacon, smoked meat, dried-up cadavers and the like in which cholesterol constitutes the only or main sterol present. *Drosophila* develops on moist food rich in carbohydrates in which yeast grows abundantly. In fact, it is generally considered that living yeast is the main food of the *Drosophila* larva [Guyénot, 1917]. It is therefore illuminating to find that the sterol requirements of *Drosophila* are fulfilled by ergosterol, the principal sterol of yeast, whereas as far as we know to-day cholesterol and 7-dehydrocholesterol, which appears to be generally associated with cholesterol, constitute the only active sterols for *Dermestes*. It will be shown in a later publication that the requirements of several flour insects for sterols are, amongst other sterols, met by sitosterol, the principal sterol of wheat germ oil.

In recent years the antirachitic effect of irradiated cholesterol on rats has been shown to be due to the presence before irradiation of 7-dehydrocholesterol as an impurity. It is, however, certain that for both *Drosophila* and *Dermestes* the presence of this impurity is immaterial, for purified cholesterol proved as effective as commercial cholesterol. The insects, then, require in their food a sterol, and it is not surprising that this requirement is not fulfilled by calciferol, in which the tetracyclic arrangement of the carbon atoms has been changed, and which is consequently not a true sterol.

So far it has been shown for at least five different insects, namely two flies, *Lucilia* [Hobson, 1935] and *Drosophila* [Van t'Hoog, 1936], three beetles, *Acanthoscelides* [Chiu & McCay, 1939], *Tribolium* [Fröbrich, 1939; Offhaus, 1939] and *Dermestes*, and for a snail *Helix pomatia* [Howes, 1937], that the inclusión of a sterol in the diet is essential for growth. This also applies to four more beetles: *Lasioderma*, *Sitodrepa*, *Silvanus* and *Ptinus*, and one moth *Ephestia* [Fraenkel, unpublished]. It seems, however, that the requirements of living organisms for sterols are of a more general occurrence than has hitherto been supposed. Cailleau [1938] has shown that the parasitic flagellates *Trichomonas columbae* and *Tr. foetus* only grow in the presence of cholesterol. A certain strain (*W*) of yeast requires for growth a sterol in addition to bios; cholesterol is inactive but sitosterol, ergosterol and calciferol are active [Devloo, 1938]. The position therefore is that invertebrates and even some micro-organisms require sterols but do not require the irradiated products of sterols (vitamin D). In the light of these findings it seems that the almost universally accepted thesis of the synthesis of cholesterol in the mammalian body might require revision. Recent evidence on this subject is conflicting [Channon & Tristram, 1937; Rittenberg & Schoenheimer, 1937], and it has only recently been pointed out by Bacharach & Drummond [1940] that the question whether mammals are able to synthesize cholesterol is entirely unsettled.

SUMMARY

1. Several methods for investigating the sterol requirements of the beetle *Dermestes vulpinus* have been described.

2. The larva of *D. vulpinus* only grows if the food contains a sterol. Active sterols include purified cholesterol (free from 7-dehydrocholesterol), cholesteryl acetate, 7-dehydrocholesterol and 7-dehydrocholesteryl monobenzoate. Sitosterol, ergosterol, zymosterol, cholestanol, cholestene, 7-ketocholesteryl acetate, 7-hydroxycholesterol, 7-hydroxycholesteryl dibenzoate and calciferol (vitamin D_2) are inactive.

3. The minimum quantity of sterol required for successful growth is about 1 mg./3 g. of food for cholesterol and 7-dehydrocholesteryl monobenzoate and less than 0·5 mg./3 g. of food for 7-dehydrocholesterol.

The authors are much indebted to Prof. I. M. Heilbron and Dr E. R. H. Jones for valuable advice and the gift of samples of purified cholesterol, calciferol, zymosterol and cholestanol, and to Dr A. R. Bacharach and Dr E. Lester Smith (Glaxo Laboratories) for the gift of samples of a number of sterols listed on p. 712.

REFERENCES

Bacharach & Drummond (1940). *Chem. & Ind.* **59**, 37.
Cailleau (1938). *C.R. Soc. Biol., Paris*, **47**, 861.
Channon & Tristram (1937). *Biochem. J.* **31**, 739.
Chiu & McCay (1939). *Ann. ent. Soc. Amer.* **32**, 164.
Cremer (1935). *Z. Untersuch. Lebensmitt.* **70**, 136.
Devloo (1938). *Arch. int. Physiol.* **46**, 157.
Dick (1937). *Ann. appl. Biol.* **24**, 762.
Fröbrich (1939). *Z. vergl. Physiol.* **27**, 335.
Gay (1938). *J. exp. Zool.* **79**, 93.
Guyénot (1917). *Bull. Biol.* **51**, 137.
Hobson (1935). *Biochem. J.* **29**, 1292.
Howes (1937). *Biochem. J.* **31**, 1489.
Offhaus (1939). *Z. vergl. Physiol.* **27**, 384.
Rittenberg & Schoenheimer (1937). *J. biol. Chem.* **121**, 235.
Van t'Hoog (1935). *Z. Vitaminforsch.* **4**, 300.
—— (1936). *Z. Vitaminforsch.* **5**, 118.

76. THE USE OF MICRO-ORGANISMS IN SUGAR ANALYSIS

3. THE EFFECTS OF VARIOUS SALTS ON THE ACTIVITY OF *PROTEUS VULGARIS* IN REMOVING GLUCOSE AND SOME POSSIBLE SOURCES OF ERROR IN ITS USE AS A REAGENT FOR THE ESTIMATION OF GLUCOSE

By THOMAS FREDERICK NICHOLSON AND
REGINALD McGREGOR ARCHIBALD

From the Department of Pathological Chemistry, University of Toronto

(*Received 31 May 1941*)

In the eight years since *Proteus vulgaris* was described as a reagent for the quantitative estimation of small amounts of glucose [Harding & Nicholson, 1933], continued experience has revealed a number of possible sources of error, and improvements in the methods of growth and use of the organism have simplified the procedure.

The effect of salt concentration

It was noted previously [Harding *et al.* 1936] that a concentration of 5 % Na_2SO_4 inhibited completely the glucose-removing action of *P. vulgaris* and that a concentration of 3 % led to a definite decrease in activity. The effects of varying concentrations of different salts which might be encountered in filtrates or hydrolysates of biological materials were determined. The results are shown in Table 1.

Table 1. *The effect of salt concentration on the glucose-removing power of* P. vulgaris

	% removal*					
Molar concentration	Na_2SO_4	K_2SO_4	NaCl	KCl	K_2HPO_4-KH_2PO_4†	Na_2HPO_4-NaH_2PO_4†
0·00	100	100	100	100	100	100
0·0075	—	—	—	—	350	—
0·015	112	130	119	142	350	231
0·02	110	128	123	158	—	—
0·03	110	124	132	188	350	231
0·05	86	119	140	184	—	—
0·07	70	104	135	180	350	230
0·15	43	63	103	148	350	164
0·20	30	52	85	114	—	74
0·25	24	43	73	89	350	21
0·35	0	32	53	63	—	—
0·45	—	31	34	55	—	—
0·55	—	31	23	50	—	—
0·65	—	30	17	46	—	—
0·75	—	29	—	—	—	—
1·50	—	—	16	44	350	20

* The amount of *P. vulgaris* used was just sufficient to remove 20 % of the glucose from 10 ml. of a 20 mg. per 100 ml. solution in 30 min. at 37°. This removal was taken as 100 for purposes of comparison.

† These mixtures were made up so that the concentrations were of 0·918 *M* of the dibasic salt and 0·588 *M* of the monobasic salt in the stock solution. The *p*H of the various dilutions ranged from 6·9 in the strongest to 7·1 in the weakest solution.

Small concentrations of all the salts tested had an accelerating effect on the action of *P. vulgaris*. Potassium salts had a greater effect than sodium salts. Chlorides were more active than sulphates. At higher concentrations there is a marked inhibition of the removal activity, complete in the case of sodium sulphate at a concentration of 0·35M and reaching a fairly constant level for each of the other salts tested: 30 % removal at concentrations of 0·35M and over for K_2SO_4: 16 % removal at concentrations of 0·65M and over for NaCl: 46 % removal at the same concentration or over for KCl. The sodium phosphate mixtures had a very similar effect, except that the activating effect of the lower concentrations was more marked. Potassium phosphate mixtures acted quite differently in that the activation was much more marked and was present throughout the range of concentrations employed.

The inhibiting effect of the higher salt concentrations has also been observed with *Gaffkya tetragena*, an organism which is used as a reagent for the removal of glucose and fructose [Nicholson, 1936], but the lower concentrations produce no increase in activity. This applies to the phosphates as well as to the other salts. No change whatever in sugar-removing power has been observed in the case of yeasts.

The marked activating power of potassium phosphate is not due in more than a minor degree to any buffering action, as is shown in Table 2.

Table 2. *Effects of phosphate, bicarbonate-carbonic acid and hydroxymethylglyoxaline hydrochloride-NaOH buffers on the activity of* P. vulgaris *in removing glucose from a solution containing 2 mg. glucose*

	No salt	KH_2PO_4-K_2HPO_4	$NaHCO_3$-*H_2CO_3	KCl	4 (or 5)-hydroxymethylglyoxaline hydrochloride + NaOH†
	—	0·03M	0·03M	0·03M	[Kirby & Neuberger, 1938]
mg. glucose removed	0·28	1·0	0·56	0·43	0·53

All fermentations were carried out at a pH of 7·0. Removal in mg. from 10 ml. of a 10 mg. per 100 ml. solution of glucose using 0·10 g. *P. vulgaris* and incubating for one half-hour at 37–38°.

* A slow stream of CO_2 was run through the solution during incubation.

† We are indebted to Dr H. O. L. Fischer of the Department of Chemistry of this University for the dihydroxyacetone from which the hydroxymethylglyoxaline hydrochloride was prepared.

The glucose-removing activity of *P. vulgaris* may be further increased by adding potassium phosphate to the media in which the organisms are grown. The following media have been used:

Phosphate broth: 4375 ml. tap water, 625 ml. phosphate solution (see below), 50 g. NaCl, 30 g. glucose, 100 g. bacto-peptone (Lemco 62). Sterilize and tube in the usual manner.

Phosphate agar: 30 g. agar, 875 ml. broth, 50 ml. phosphate solution (see below), 10 g. glucose. Sterilize and bottle.

The potassium phosphate may be most conveniently added during the course of the dilution of the solution to be analysed for glucose. If, however, a stock 1·5M phosphate mixture (16 g. $K_2HPO_4 + 8$ g. KH_2PO_4 in 100 ml. water) is used, the dilution (0·5 %) involved in adding the potassium phosphate to the actual solution being fermented is negligible.

The batches of *P. vulgaris* grown on such media showed 60 % greater activity than did those grown on the media originally described [Harding & Nicholson, 1933]. When *P. vulgaris* grown on phosphate-containing media is used on glucose solutions containing potassium phosphate (0·0075M), the glucose-removing power of the organisms is so increased that 0·5 g. of wet-weight packed organisms will remove 2·5 mg. of sugar by incubation for 10 min. at 37°. When grown on the original media and acting without phosphate added to the glucose-containing solution it takes 0·5 g. wet-weight packed bacteria and incubation for half an hour at 37° to remove 1 mg. of glucose. These procedures have no effect on the specificity of *P. vulgaris* for glucose.

The accelerating action of phosphates on yeast juices and maceration extracts is well known, as is the effect of higher concentrations in yeast-juice fermentation in delaying the rate of CO_2 production [Harden & Young, 1908; Harden, 1932]. Meyerhof [1918] noted the retarding effect of salts other than phosphates and Harden & Henley [1921] observed that sulphates had a greater retarding effect than chlorides. In the case of *Proteus*, however, the accelerating effect of phosphate is noted in the intact organism, not in its extracts only as in the case of yeast. Most of the observations on the retardation effects of salts on yeast have been made on extracts, but Lampe & Kilk [1931] noted that the NaCl formed by the neutralization of HCl used in the inversion of cane sugar decreased the alcohol yield on fermentation. The action of *P. vulgaris*, however, is retarded more by salts of sodium than by those of potassium, whereas yeast extracts are affected equally by either [Harden & Henley, 1921]. In the case of *P. vulgaris* there is also an accelerating effect by small concentrations of chlorides and sulphates as well as phosphates and the potassium phosphate has no retarding effect even in relatively high concentrations.

The effect of p*H*

It was previously reported [Harding & Nicholson, 1933] that *P. vulgaris* would not act on glucose at a *p*H lower than 6·5 or higher than 8·0. It has been found, however, that it is inadvisable to use this micro-organism at any *p*H higher than 7·2 because, although the bacteria are still active in removing glucose in the more alkaline solutions, there is a small amount of lysis with the liberation of varying amounts of reducing substances. If *Proteus* alone is being used this does not affect the accuracy of the glucose estimation, provided that the blank is incubated at the same *p*H as the solution being tested, but if a differential fermentation is performed it is essential that the reaction be kept below *p*H 7·2, preferably between *p*H 6·5 and 7·0 because some of the products of the bacteriolysis are acted on by yeasts to give reducing products. This action is illustrated in Table 3.

Table 3. *Action of* P. vulgaris *followed by* Monilia Krusei *on a solution containing 4 mg./100 ml. glucose and 4 mg./100 ml. fructose* (a) *in neutral solution* (p*H 7·0*), (b) *in alkaline solution* (p*H 7·8*)

	P. vulgaris			*P. vulgaris* followed by *M. Krusei*		
Total reduction	Blank	Reduction after fermentation	Removal	Blank	Reduction after fermentation	Removal
(*a*) 0·69	0·00	0·34	0·35	0·02	0·02	0·34
(*b*) 0·69	0·09	0·43	0·34	0·17	0·72	Added 0·21

The reducing values were determined using the Harding & Downs [1933] reagent. 1 ml. of reagent and 1 ml. of sugar solution were used. The results are expressed as ml. of 0·005 *N* $Na_2S_2O_3$.

This phenomenon is of particular importance in solutions containing any considerable amount of urea, because *P. vulgaris* will produce sufficient ammonia from the urea to make the solutions quite alkaline. Such solutions must therefore be well buffered before the introduction of *P. vulgaris*, or preferably be freed from urea.

The effect of the presence of nitrates

P. vulgaris converts nitrates into nitrites. The nitrites will liberate I_2 from KI in acid solution and therefore interfere with any iodimetric sugar estimation. The sugars may be obtained free from nitrates and other interfering substances by the modified copper-lime technique [Harding *et al.* 1936], or the nitrites may be converted into NH_3 by saturating the solution with H_2S and shaking vigorously in presence of air for a few minutes. The H_2S is then removed by aeration; the NH_3 is removed by the use of KH_2PO_4 and MgO [Harding & Downs, 1933].

Some modifications in the methods of using P. vulgaris

The great enhancement of the glucose-removing power of *P. vulgaris* in the presence of potassium phosphate together with the use of Lloyd's reagent for final clearing of the *P. vulgaris* from the fermented solutions after centrifuging [Griffiths & Waters, 1936] has led to the adoption of the following procedure. The organisms are washed off the phosphate agar surface, packed in the angle centrifuge, then rewashed once in distilled water. A 25 % suspension of the wet packed bacteria is made in distilled water. 2 ml. of the suspension (containing 0·5 g. *P. vulgaris*) are taken for each 2·0 mg. of glucose to be removed and the organisms are packed and dried as described by Harding & Nicholson [1933]. Add to each 10 ml. of the sugar solution to be tested, preferably during dilution, 0·05 ml. of a solution of potassium phosphate:

$$\left.\begin{matrix} K_2HPO_4\text{—16 g.} \\ KH_2PO_4\text{—8 g.} \end{matrix}\right\} \text{in 100 ml. } H_2O.$$

Incubate in the water bath at 37–38° for 10 min. and centrifuge. Clear the few remaining organisms from the supernatant fluid by shaking with a little Lloyd's reagent and filtering through a fluted filter paper [Griffiths & Waters, 1936].

If no angle centrifuge is available the following procedure may be followed. Wash the bacteria from agar, place not more than 7 ml. of the suspension in a 15 ml. conical centrifuge tube and centrifuge at 2500 revolutions per min. for 15 min. Pour off the cloudy supernatant fluid. Add 5 ml. of distilled water, resuspend and recentrifuge. Pour off cloudy supernatant and recentrifuge for 15 min. Make up a 50 % suspension of the bacteria. Put not more than 0·5 ml. of the suspension (sufficient to remove 1 mg. glucose in 10 min.) in a 15 ml. centrifuge tube, centrifuge for 15 min., pour off the supernatant fluid and recentrifuge for 5 min. Pour off any fluid remaining and dry the sides of the centrifuge tube. Incubate with the solution to be tested (containing not more than 1 mg. glucose) in the presence of 0·0075*M* phosphate. After incubation centrifuge for 15 min. Clear the markedly cloudy supernatant fluid by shaking with 1·2 g. Lloyd's reagent and filtering.

SUMMARY

The ability of *P. vulgaris* to remove glucose from solution is enhanced by small concentrations of NaCl, Na_2SO_4, KCl and K_2SO_4 and is greatly inhibited by larger concentrations.

The potassium salts are the more active accelerators and sodium salts are the stronger inhibitors. Sulphates show a greater inhibiting effect than do chlorides.

Sodium phosphate in small concentrations and potassium phosphate in all concentrations greatly enhance the glucose-removing ability of the organisms, potassium phosphate increasing the glucose removal 3·5 times.

Fermentation in a solution more alkaline than *p*H 7·2 introduces errors due to the dissolving of reducing substances from the cells.

The reduction of nitrates to nitrites by *P. vulgaris* may lead to errors in iodimetric titrations. Methods for obviating this difficulty are described.

A method for using *P. vulgaris* without an angle centrifuge is given.

REFERENCES

Griffiths & Waters (1936). *Amer. J. Physiol.* **117**, 134.
Harden (1932). Alcoholic Fermentation. London: Longmans Green and Co.
—— & Henley (1921). *Biochem. J.* **15**, 312.
—— & Young (1908). *Proc. roy. Soc.* B, **80**, 299.
Harding & Downs (1933). *J. biol. Chem.* **104**, 11.
—— & Nicholson (1933). *Biochem. J.* **27**, 1082.
—— —— & Archibald (1936). *Biochem. J.* **30**, 326.
Kirby & Neuberger (1938). *Biochem. J.* **32**, 1146.
Lampe & Kilk (1931). *Brennereiztg.* **48**, 121.
Meyerhof (1918). *Hoppe-Seyl. Z.* **102**, 1.
Nicholson (1936). *Biochem. J.* **30**, 1804.

77. CORRELATION BETWEEN SEX HORMONES, THYROID HORMONES AND DESOXYCORTICOSTERONE AS JUDGED BY THEIR EFFECTS ON THE WEIGHTS OF ORGANS OF GONADECTOMIZED RATS

By V. KORENCHEVSKY and K. HALL

From the Lister Institute, London[1]

(*Received 31 May 1941*)

THAT there is a correlation between the endocrine secretions of the adrenal, thyroid and sex glands has repeatedly been suggested in the literature on indirect evidence [see survey of literature, Cramer, 1928; Harington, 1933; Grollman, 1936; Means, 1937; Crotti, 1938]. At the same time, the adrenal, thyroid and sex glands are considered to play some part in a manifold mechanism responsible for the maintenance of physical and mental vigour of the organism.

Therefore an investigation of the interrelation of the hormones secreted by these glands is desirable not only for theoretical reasons, but also for possible practical purposes, since these hormones are already extensively used, although not always on sufficiently scientific grounds, in the treatment of different pathological conditions.

The effect of these hormones, administered separately, on the weights of some organs has been investigated extensively in the case of sex and thyroid hormones, but very little in the case of desoxycorticosterone. In order to avoid repetition, a brief survey of this literature will be given for each hormone in the paragraphs dealing with the respective hormones. As far as we know, the correlative changes in weight and structure of organs produced by these three groups of hormones, sex, thyroid, and corticoadrenal, when administered simultaneously, have not been studied.

In order to elucidate this problem, we undertook a number of experiments, some of which are still in progress. In this paper we shall include only some of the results of the first series of experiments, namely, changes in the weights of the organs. The results of their histological examination will be published elsewhere.

Technique

For the experiments 168 rats were used, 86 males and 82 females. Ten rats were kept as normal controls, and all the remaining animals were gonadectomized when 22–24 days old. The final average age of the rats varied from 167 to 207 days. The distribution of the rats into the different groups, their number in each group, the final body weight and the weights of those organs which were changed in at least some of the groups are given in Tables 1 and 2. All animals were killed by bleeding.

---

[1] Dr Korenchevsky desires to express to Oxford University, to Prof. E. S. Goodrich and to Prof. J. A. Gunn his own and the Lister Institute's gratitude for the kind hospitality extended to him and his co-workers in the Department of Zoology and Comparative Anatomy and the Nuffield Institute for Medical Research.

Table 1. *The effects of male, oestrogenic and thyroid hormones and desoxycorticosterone, administered alone or in combination, on actual weights of organs of castrated rats*

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
			Castrated rats treated with hormones												
	Control rats		Thyroid hormones		Testosterone propionate			Androsterone			Desoxycorticosterone acetate			Oestradiol B.B. 0·09 mg.	
						+Thyroid hormones			+Thyroid hormones			+Thyroid hormones			
	Normal	Castrated	Small dose	Large dose	Alone	Small dose	Large dose	Alone	Small dose	Large dose	Alone	Small dose	Large dose	Alone	+Thyroid hormones small dose
Seminal vesicles (mg.)	1049	13	13	15	1298	1299	1052	61	38	51	14	12	17	80	96
Prostate (mg.)	1394	60	61	67	1382	1133	1217	188	169	185	62	72	62	105	122
Penis (mg.)	357	84	80	70	348	353	336	183	157	150	75	91	63	79	77
Preputial glands (mg.)	95	30	34	31	108	114	99	66	68	73	30	36	38	40	42
Adrenals (mg.)	54	58	58	93	50	49	74	47	47	70	49	53	87	70	88
Hypophysis (mg.)	13·1	15·9	14·2	15·1	14·6	13·4	13·6	13·2	14·4	14·3	16·1	18·3	15·4	41·5	47·7
Thymus (mg.)	167	224	222	111	96	114	117	185	220	118	232	266	122	132	156
Liver (g.)	11·28	8·65	8·36	10·58	10·24	10·17	10·63	9·55	9·73	9·87	9·44	11·73	10·90	8·76	11·09
Kidneys (g.)	2·24	1·66	1·61	2·37	2·03	2 01	2 79	1·83	1·91	2·34	1·90	2·27	2·87	1·82	2·47
Spleen (mg.)	645	848	807	690	738	836	739	687	889	658	702	1324	709	733	885
Heart (mg.)	1101	943	982	1398	1084	1126	1419	1043	1118	1290	999	1270	1437	901	1070
Retroperitoneal fat (g.)	7·3	9·5	8·3	1·7	7·1	9·6	2·2	11·8	10·9	1·1	10·8	11·3	1·1	11·1	7·7
Final body weight (g.)	334	293	272	258	313	319	287	324	313	248	306	350	254	257	277
No. of rats in group	4	12	8	3	9	7	3	7	6	3	7	5	2	5	5

Note. In both tables, all the 'small dose' groups belong to the first experiment, the 'large dose' groups to the second experiment, and they should be compared accordingly.

Table 2. *The effects of male, oestrogenic and thyroid hormones, administered alone or in combination, on actual weights of organs of ovariectomized rats*

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
			Ovariectomized rats treated with hormones										
	Control rats		Thyroid hormones		Androsterone			Oestradiol B.B. 0·015 mg.			Oestradiol B.B. 0·015 mg.		
						+Thyroid hormones							
	Normal	Ovariectomized	Small dose	Large dose	Alone	Small dose	Large dose	Alone	+Thyroid hormones small dose	—Androsterone + thyroid hormones small dose	Alone	+Thyroid hormones large dose	+Androsterone + thyroid hormones large dose
Uterus (mg.)	409	31	39	31	36	40	28	191	220	240	207	238	368
Vagina (mg.)	292	160	187	178	236	224	234	273	304	333	255	307	365
Preputial glands (mg.)	74	49	53	52	131	116	161	48	44	123	49	49	212
Adrenals (mg.)	63	62	63	103	51	52	85	55	84	77	98	166	149
Hypophysis (mg.)	15·1	14·5	14·7	13·4	16 1	13·8	14·5	13·8	15·8	15·4	20·2	20·5	18·8
Thymus (mg.)	136	184	228	192	194	170	137	125	158	133	167	176	152
Liver (g.)	6·66	7·22	7·94	9·53	9·08	9·44	10·75	5·97	7·27	8·21	9·03	13·20	11·70
Kidneys (g.)	1·58	1·53	1·67	2·41	1·75	1·81	2·44	1·34	1·69	1·84	1·79	2·98	3·05
Spleen (mg.)	479	838	788	838	595	783	1564	460	797	978	581	1053	906
Heart (g.)	795	828	953	1158	941	1055	1345	783	907	984	921	1383	1247
Abdominal fat (g.)	17·3	10·4	12·4	0·5	12·8	15·7	1·0	12·0	9·8	17·0	9·0	1·0	2·3
Final body weight (g.)	218	256	262	213	289	288	247	220	223	262	227	233	247
No. of rats in group	6	11	10	3	9	6	3	5	5	6	3	3	3

Only those hormones which are naturally present in the organism were used (administered, however, mostly as esters). The following hormones were given, in the doses indicated per week: in experiments on males—androsterone 4·5 mg., testosterone propionate 0·750 mg. and oestradiol 3-benzoate-17n-butyrate 0·09 mg. (abbreviated in the paper to 'oestradiol B.B.'); in experiments on females—androsterone 4·5 mg., oestradiol B.B. 0·015 mg. In one experiment (Table 2, cols. VIII–X) this oestrogen was injected only for the first 3 weeks; in the second experiment (cols. XI–XIII) for the first 3 weeks, and also during 2 weeks towards the end of the experiment.

Thyroxine, thyroid powder and desoxycorticosterone were administered to both sexes; the thyroid powder was given by pipette in gum acacia suspension sweetened with honey. The groups receiving a 'small dose' of thyroid hormones were given 30–40 mg. of thyroid powder (Parke, Davis and Co.) three times weekly; the duration of this experiment was 54–58 days. The groups receiving the 'large dose' were fed with 40 mg. three times weekly for the first 3 weeks, 80 mg. three times weekly for the next 6 weeks, and 80 mg. five or six times weekly for the last 2 weeks; this group also received during the last 3 weeks of the experiment 1 mg. injected subcutaneously one to three times weekly (total 9 mg. per rat) of thyroxine sodium (British Drug Houses). The variation in the dose of thyroid hormones was dependent upon the loss in weight and appetite, in order not to impair too much the general condition of the rats. The duration of the 'large dose' experiment was 76 days.

The rats received desoxycorticosterone in the form of two subcutaneously implanted crystalline pellets, one of acetate (50 mg.) and the other of pure hormone (25 mg.). The group of rats receiving the large dose of thyroid received also in addition to the two implanted pellets of desoxycorticosterone, subcutaneous injections of 2–4 mg. weekly of desoxycorticosterone acetate in oil. No difference was observed in the weights of the organs of these rats as compared with those of rats receiving desoxycorticosterone in the form of pellets only. Therefore in Table 1, the data of these two groups have been pooled.

For the same reason the data from the two experiments have been pooled in the tables in the case of control rats or rats injected with testosterone propionate alone.

In other respects the conditions of the experiments were the same as those described in our previous papers. The diet was similar to that previously described [Hall & Korenchevsky, 1938] except that fish was not always obtainable, and the yeast used, although obtained from the same source, was poorer in B-vitamins.

The weights of the organs were also calculated per unit of body weight, but for economy of space the results are not tabulated here. When of importance, they will be discussed in the text.

Effect of gonadectomy and of sex hormones

In both males and females the effects of gonadectomy (Tables 1 and 2, col. II), male sex hormones (Table 1, cols. V and VIII; Table 2, col. V) and oestrogens (Table 1, col. XIV; Table 2, cols. VIII and XI) were similar to those described in our previous papers [1933–41].

Thus, the *body weight* of castrated males was less, and of ovariectomized females was greater than that (col. I) of the respective normal controls. In both sexes the male hormones, in the comparatively small doses used, caused a slight increase in the body weight when compared with gonadectomized controls. Oestrogens, on the other hand, decreased the gain in body weight, as has been noted several times in our own papers and in those of other workers. That there was only a slight depression of growth in the present experiments is explained by the use of the butyrate-benzoate ester of oestradiol, the effect of which is mild if sufficiently long intervals (in this case, at least 1 week) are allowed between the injections.

Fat deposition. The same explanation applies to the absence of any pronounced depression of fat deposition by oestradiol benzoate-butyrate (Table 2, col. XI), or even to a slight increase (Table 1, col. XIV; Table 2, col. VIII). Usually, as our previous experiments have shown, oestrogens depress fat deposition. In both sexes, androsterone caused a slight increase and testosterone propionate a slight decrease in the amount of fat deposited.

In our earlier papers we have shown that weak male hormones such as androsterone, or small doses of strong hormones cause no change or a slight increase in the amount of fat deposited; while medium doses and, especially, large doses of strong male hormones decrease the amount of adipose tissue in the organism. As has been emphasized previously [e.g. Korenchevsky, Dennison & Eldridge, 1937, 1, p. 469; 1937, 2, p. 478; Korenchevsky, Dennison & Hall, 1937, 1, p. 782; Korenchevsky, Hall & Ross, 1939, p. 214, Table 2, col. XIII) it often happens that after the administration of male hormones, while (as compared with the gonadectomized controls) fat deposition is decreased, the body weight remains unchanged or is even increased. In the present experiments this occurred in the case of testosterone propionate (Table 1, col. V). These observations appear to be important, since they suggest that the sex hormones may have anabolic properties. Thus, the increased gain in body weight is probably to be explained by an increase in the anabolic processes of building new tissues (e.g. in liver, kidneys, heart, among others) and not by a passive accumulation of fat in the organism. Instances indicating catabolic properties of large doses of oestrogens and male hormones have been given in our previous papers.

Sex organs. The usual return to or towards normal was observed, the degree of change depending upon the strength of the compound and the size of the dose. The specific effect of oestrogens on male sex organs has been fully discussed and the literature reviewed in our previous papers [e.g. Korenchevsky, 1941]. We wish to emphasize here only one detail of the effect of androsterone (a hormone naturally present in females) on the female sex organs, in confirmation of our previous observation [Korenchevsky, Hall & Burbank, 1939]. Lengthening the period of injection of this hormone increases the hypertrophy of the vagina and female preputial glands, but not of the uterus (Table 2, col. V).

Adrenals. The literature has been reviewed by Hall & Korenchevsky [1938] and Hall [1940]. In the present experiments, no effect of gonadectomy was noticed, but as before male hormones caused a decrease in weight and size of the glands, the mild effect being due to the comparatively small doses used. As before, the effect of oestrogen was not constant, but in those groups in which it was injected for a longer period (Table 2, col. XI) or in a larger dose for a long period (Table 1, col. XIV) the adrenals were definitely enlarged. Histological observations [Hall, 1940] suggest a possible explanation of these variations. Hyperaemia in the adrenals of animals treated with oestrogens varies in degree, in some cases being very pronounced. Our rats were killed by bleeding. The varying degree of hyperaemia, and the emptying of the hyperaemic areas by bleeding would have a considerable effect upon the weight of the organ.

The hypophysis was hypertrophied after prolonged injections of small doses (Table 2, col. XI) and large doses (Table 1, col. XIV) of oestrogens. In males there was a slight 'castration' hypertrophy of the gland (Table 1, col. II) which was restored to normal by injections of androsterone (col. VIII). All these data are in agreement with our previous results [e.g. Korenchevsky, Hall & Ross, 1939].

Thymus. The literature has been reviewed and the results of our previous experiments summarised by Ross & Korenchevsky [1941]. The thymus, which is always enlarged after gonadectomy, decreases in size and weight after injections of male hormones and oestrogens. In the present experiments the only exception to this rule was the absence of the usual response of the thymus to injections of androsterone in females (Table 2, col. V). This sometimes happens after treatment with weak hormones or small doses of strong hormones.

Liver, kidneys and heart. Again our present data confirm our previous results, showing hepato-, nephro- and cardio-trophic properties of male hormones. Thus a slight atrophy of these organs is obtained after castration in males (Table 1, col. II) and a return towards normal after injections of male hormones (Table 1, cols. V and VIII) but not of oestrogens. Gonadectomy (as also in these experiments) does not usually have an atrophying effect on these organs in females. Still, the hepato-, nephro- and cardio-trophic properties of male hormones are clearly demonstrated also by the effects of the injections into gonadectomized females in which the liver, kidneys and heart become larger than in the intact or gonadectomized uninjected controls (compare Table 2, col. V with cols. I and II).

In rats of the same breed, kept under the same conditions and on the same diet, the liver, kidneys and heart are larger in males than in females. Therefore in our previous paper we defined the action of male hormones on the liver, kidneys and heart of females as a masculinizing effect [Korenchevsky, Hall Burbank & Cohen, 1941, p. 398].

As before, the effect of oestrogens on the actual weights of these organs was not constant, but a regular enlargement of liver, kidneys and heart is always shown in the relative weights. If, however, such mild and less toxic oestradiol esters as the benzoate-butyrate are used with sufficiently long intervals between the injections, it is sometimes possible to obtain hypertrophy of these organs even in actual weight, as happened, for instance, in one of the present experiments (Table 2, col. XI). These facts suggest that under certain conditions, and in the right doses, oestrogens might also possess non-sexual stimulating properties similar to those of male hormones.

All the data in our previous papers [1933–9] demonstrating the hepato-, nephro- and cardio-trophic properties of sex hormones have recently been summarized and examined in detail [Korenchevsky & Ross, 1940; Korenchevsky, Hall, Burbank & Cohen, 1941]. The nephrotrophic property of testosterone propionate has also been investigated and confirmed by Selye [1939; 1940], Ludden *et al.* [1941] and Kochakian [1941], the latter also confirming the hepato- and cardio-trophic effects.

Spleen. As found in our previous experiments, the effects of gonadectomy and of sex hormones on this organ are not constant, but in some of the experiments there were pronounced and definite changes. A detailed investigation of the data from all our experiments will be published elsewhere.

Effect of thyroid hormones

Cameron [1930] and Cameron & Carmichael [1920; 1921; 1922; 1924; 1926] give an account of the literature and describe their own numerous and important experiments.

The thyroid hormones administered by mouth in the form of dried thyroid powder produce hypertrophy of adrenals, liver, kidneys, heart and, less constantly, of spleen and lymphatic glands, loss of fat tissue, decrease of the growth rate or, with large doses, actual loss of body weight of animals. Four to seven weeks after thyroid feeding has been stopped [Cameron & Carmichael, 1922], the hypertrophied organs usually return to normal, while the animal's growth rate becomes accelerated above normal to such an extent that the weights of the treated rats usually exceed those of the controls.

Thyroxine, although found to possess every known activity of thyroid secretion, has not so far been proved with certainty to contain the maximum of thyroid activity. The best definition of the question is given by Harington [1933, p. 169]: 'it therefore seems unnecessary to assume the existence in the thyroid of any specifically active principle other than thyroxine, so long as we are careful to bear in mind that thyroxine is not to be regarded as itself the complete active principle, but is rather to be considered as the constituent which confers specific physiological properties upon a more complex molecule'. According to Cameron & Carmichael [1921] thyroxine administered by mouth produces hypertrophy of the same organs as in rats fed with thyroid powder. Sternheimer [1939], injecting thyroxine, confirmed the hypertrophy of the liver, and Uotila [1940] of adrenals. Uotila found no definite change in the weight of the testes, but a decrease of about 40% in the weight of the seminal vesicles.

In the present experiments the small dose of thyroid hormones had practically no effect in males and very little in females (Tables 1 and 2, col. III). This 'small dose' is to be considered comparatively large for rats, and the lack of response to this dose in our experiments can probably be explained by the richness of our diet in vitamin A. This vitamin has been shown to be antagonistic to the thyroid hormones and therefore more or less neutralizes their effects (see the Survey of Literature and their own experiments, Smith & Perman [1940]). With the large dose we obtained the usual effects, thus confirming the results recorded by previous authors: the growth rate, final body weight and fat deposition were decreased; adrenals, liver, kidneys, heart and, not constantly, spleen were hypertrophied. No effect was found on the weight and size of the sex organs and hypophysis of gonadectomized rats (Tables 1 and 2, col. IV). The large dose produced a definite atrophying effect on the thymus only in males. When the weights of the hypertrophied organs of gonadectomized rats treated with the large dose of thyroid hormones are compared with those of normal untreated rats, it is found that they often considerably exceeded normal level, especially in females.

Effect of desoxycorticosterone

According to Selye [1940] and Ludden *et al.* [1941], desoxycorticosterone causes enlargement of the kidneys and [Hooker & Collins, 1940] has some stimulating activity on male sex organs.

In the present experiments (Table 1, col. XI) slight hypertrophy of liver and kidneys was found, but no definite effect was noticed on the other organs examined, or on fat deposition or body weight.

Similar small changes have been obtained in another experiment of 21 days' duration (not included in the Tables) on 11 young normal males (final age 52 days): the weights in the control rats were—liver 12·0 g., kidneys 2·3 g., heart 0·949 g.; and in the injected rats—liver 12·91 g., kidneys 2·53 g., heart 1·080 g.

Correlation between the male sex and thyroid hormones

Males. When castrated rats were treated simultaneously with both thyroid and sex hormones, the effect obtained (Table 1, cols. VI, VII, IX, and X) did not exceed that produced by each hormone separately in the case of thymus, liver, kidneys, spleen, heart and fat deposition. Small changes in the weights of sex organs and hypophysis are not conclusive, unless confirmed on a larger number of animals, or unless histological examination shows some effects.

A definite neutralizing or antagonistic effect was found on adrenals: the hypertrophy of this gland produced by large doses of thyroid hormones (weight of adrenals 93 mg.) was lessened by the addition of testosterone propionate (74 mg.) or androsterone (70 mg.). As has been emphasized in our previous papers, this action on adrenals is a typical property of male hormones.

Another effect of male hormones antagonistic to that of thyroid hormones was shown in the gain in body weight: when thyroid hormones were administered alone, the gain was 27 g. with the small dose and 7 g. with the large dose; when testosterone propionate was injected simultaneously, the respective figures were 76 and 32 g. The neutralizing effect of androsterone was considerably less pronounced, the gain being 38 and 10 g. respectively, i.e. androsterone was unable to neutralize the effect of the large dose of thyroid hormones. It should be emphasized, however, that two rats of this group suffered from slight pneumonia, and perhaps the experiments now in progress on a larger number of animals will give more definite results. This can reasonably be expected when one considers the results on females described below.

Females. The antagonistic action of androsterone on the effect of thyroid hormones on adrenals and gain in body weight of females (Table 2, cols. VI and VII) was similar to that of testosterone propionate in males. For example, the gain in body weight of females treated with thyroid hormones alone was 24 g. (small dose) and 5 g. (large dose), while with simultaneous injections of androsterone these figures were 39 and 20 g. respectively.

As in males, there were no correlative changes in the weights of the sex organs, hypophysis and fat deposition in females.

A smaller thymus and larger spleen in rats treated with androsterone and the large dose of thyroid must be interpreted with caution, since the number of animals used was small, and the weights of all lymphatic organs sometimes vary considerably.

In females, unlike males, liver, kidneys and heart were in most cases larger than in rats receiving either hormone separately. When calculated per unit of body weight, however, these differences are less definite, so that in the case of these organs a co-operative stimulating activity can be suggested only with reservation.

Correlation between oestrogens and thyroid hormones

In contrast to the poor correlation with male hormones, thyroid hormones have a manifold interrelation with oestrogens as shown by both actual (Table 1, col. XV; Table 2, cols. IX and XII) and relative weights of several organs. The organs affected are the same in both sexes. The degree of correlation, however, differs in different organs and with different doses.

A weak co-operative activity, leading to slightly increased hypertrophy of the organs, seems to be present in some of the sex organs, the seminal vesicles and prostate of males, and the uterus and vagina of females. There is, however, no stimulating effect on the penis of males, or the preputial glands of either sex.

Because of this weak co-operative activity on the sex organs, it will be necessary to confirm the conclusions by histological investigation.

Pronounced co-operative activity of thyroid hormones and oestrogens is shown in both sexes by greater hypertrophy of adrenals, liver, kidneys and heart in rats treated with both hormones as compared with those given either hormone separately (compare in Table 1, col. XV with cols. III and XIV and in Table 2, col. XII with cols. IV and XI); or by the return to normal of the liver, kidneys and heart, the development of which is depressed by oestradiol alone (compare in Table 2, col. IX with cols. III and VIII). The fact that there is a greater loss of fat, and a decrease in the growth rate or a greater loss of body weight when both hormones are given together than when each is given separately, can also be considered as a form of co-operative activity.

The weight of the thymus remains unchanged or is even slightly increased, while the spleen, which becomes smaller after oestrogen treatment, is enlarged when both hormones are given together.

Correlation between oestrogens, thyroid hormone and androsterone in females

Since these three groups of hormones are naturally present in the female organism, simultaneous administration of these compounds to gonadectomized females was undertaken, in one experiment using a small dose (Table 2, col. X) and in another experiment a large dose (col. XIII) of thyroid hormones.

In both experiments better development of the uterus and vagina, and in one experiment (col. XIII) of the preputial glands was obtained. It is noteworthy that this co-operative activity was considerably more pronounced when the larger dose of thyroid hormones was used (col. XIII) although thyroid hormones when injected alone (cols. III and IV) have no definite effect on the weight of the sex organs.

When the small dose of thyroid hormones was combined with androsterone and oestrogens, a slightly better development of liver and kidneys was obtained which, however, did not reach those of androsterone-injected (col. V) rats. As this was not shown by the relative weights of these organs, however, the enlargement must be ascribed at least in part to the greater body weight of the animals of this group.

As shown above, decrease of growth rate or loss of body weight and loss of fat tissue, and also hypertrophy of adrenals are considerably intensified when oestrogens and thyroid hormones are administered simultaneously. Androsterone, when given in combination with these two compounds, had only a weak neutralizing (antagonistic) effect, especially when the large dose of thyroid was used. In the case of adrenals, this neutralization was insignificant. As described above, however, the neutralizing effect of androsterone was considerable when it was given in combination with thyroid hormones alone. This indicates that, as with every regulating mechanism, there are limits beyond which the regulating adjustment becomes inefficient.

Correlation between desoxycorticosterone and thyroid hormones in males

As shown in Table 1, cols. XII and XIII, no definite correlative changes were noticed in the sex organs, adrenals, hypophysis and thymus of rats injected with these two hormones.

Some neutralizing effect of desoxycorticosterone, similar to that of male hormones, on the changes produced by small doses of thyroid was present in the case of gain in body weight and fat deposition, which showed an improvement when compared with the effects of the small dose of thyroid hormones alone (Table 1, col. III). However, this neutralizing effect did not appear when the large dose of thyroid hormones was used in combination with desoxycorticosterone (col. XIII).

A definite co-operative activity, especially with the small dose of thyroid hormones, was present in the case of liver, kidneys, spleen and heart. These organs were considerably larger when both hormones were combined than when either was given separately.

Before summarizing our results, it must be emphasized that all our statements are valid only for the correlative changes in weight of the organs. Histological and chemical investigation and new experiments approaching the problem from a different angle will make clearer and more precise the interrelation of the hormones.

SUMMARY

1. Ten intact normal and 176 gonadectomized male and female rats were used in order to investigate the possible correlation between the sex and thyroid hormones and desoxycorticosterone, as judged by changes in the weights of the organs.

2. The changes produced by gonadectomy and the injections of sex hormones described in our previous papers were confirmed, including hepato-, nephro- and cardio-trophic properties of male sex hormones and, possibly, of some oestrogenic compounds.

3. The effects of thyroid hormones found previously by other workers were also confirmed. Our large dose produced decreased gain in body weight, loss of fat tissue, hypertrophy of adrenals, liver, kidneys and heart.

4. The small dose of thyroid hormones used in our experiments, although producing only slight or no changes when administered alone, yet had definite and sometimes very pronounced effects, co-operative with or antagonistic to the other hormones in several instances.

5. Desoxycorticosterone when injected alone produced only slight hypertrophy of the kidneys (confirming the results of previous workers) and of the liver.

6. The co-operative activity of thyroid hormones with oestrogens was manifested in a slightly increased hypertrophy of some of the sex organs. In females this co-operative effect was enhanced when a third hormone naturally present in females, namely androsterone, was given simultaneously.

7. Another form of co-operative activity was shown by an increase of the hypertrophy produced by thyroid hormones in liver, kidneys and heart, when oestrogens (in both sexes), or desoxycorticosterone (in males) or possibly male hormones (in females) were administered simultaneously with the thyroid hormones.

8. Co-operative activity between oestrogens and thyroid hormones was also apparent in the increased size and weight of adrenals, slower growth rate or greater loss of body weight and greater loss of fat tissue when the two hormones were combined than when each was given alone.

9. Antagonistic (neutralizing) activity of male hormones appeared in a return towards normal level of the gain in body weight and fat deposition depressed by thyroid hormones alone or in combination with oestrogens. Testosterone propionate was more effective in this respect than androsterone.

10. Another effect of androsterone and testosterone propionate antagonistic to thyroid hormones was shown in some reduction of the hypertrophy produced

in the adrenals by thyroid hormones, but this neutralizing property of androsterone was apparently ineffective in reducing the greatest degree of adrenal hypertrophy produced by simultaneous injections of oestrogens and thyroid hormones.

Grants from the Medical Research Council and the Lister Institute have enabled us to carry out this work and to them our thanks are due. We are much indebted to Messrs Ciba Ltd., in particular to Dr K. Miescher, for the generous supply of the hormones, and also to Messrs Parke, Davis and Co. for the supply of thyroid powder.

REFERENCES

Cameron (1930). *Canad. med. Ass. J.* **22**, 240.
—— & Carmichael (1920). *J. biol. Chem.* **45**, 69.
—— —— (1921). *J. biol. Chem.* **46**, 35.
—— —— (1922). *Trans. roy. Soc. Can.* **16**, 57.
—— —— (1924). *Trans. roy. Soc. Can.* **8**, 105.
—— —— (1926). *Trans. roy. Soc. Can.* **20**, 1.
Cramer (1928). Fever, heat regulation, climate and the thyroid-adrenal apparatus. London: Longmans, Green and Co.
Crotti (1938). Diseases of the thyroid, parathyroids and thymus. 3rd ed. London.
Grollman (1936). The adrenals. London: Ballière, Tindall and Cox.
Harington (1933). The thyroid gland, its chemistry and physiology. London.
Hall (1940). *J. Path. Bact.* **51**, 75.
—— & Korenchevsky (1937–38). *J. Physiol.* **91**, 365.
—— —— (1938). *Brit. med. J.* **1**, 438.
Hooker & Collins (1940). *Endocrinology*, **26**, 269.
Kochakian (1941). *Endocrinology*, **28**, 478.
Korenchevsky (1941). *J. Path. Bact.* **52**, 268.
—— Burbank & Hall (1939). *Biochem. J.* **33**, 366.
—— & Dennison (1934). *Biochem. J.* **28**, 1474.
—— —— (1936). *Biochem. J.* **30**, 1514.
—— —— & Brovsin (1936). *Biochem. J.* **30**, 558.
—— —— & Eldridge (1937, 1). *Biochem. J.* **31**, 467.
—— —— —— (1937, 2). *Biochem. J.* **31**, 475.
—— —— & Hall (1937, 1). *Biochem. J.* **31**, 780.
—— —— —— (1937, 2). *Biochem. J.* **31**, 1434.
—— —— & Kohn-Speyer (1933, 1). *Biochem. J.* **27**, 557.
—— —— —— (1933, 2). *Biochem. J.* **27**, 1506.
—— —— & Levy Simpson (1935). *Biochem. J.* **29**, 2534.
—— & Hall (1939). *Brit. med. J.* **1**, 4.
—— —— (1940). *J. Path. Bact.* **50**, 295.
—— —— & Burbank (1939). *Biochem. J.* **33**, 372.
—— —— —— & Cohen (1941). *Brit. med. J.* **1**, 396.
& Ross (1939). *Biochem. J.* **33**, 36.
—— —— & Ross (1939). *Biochem. J.* **33**, 213.
—— & Ross (1940). *Brit. med. J.* **1**, 645.
Ludden, Krueger & Wright (1941). *Endocrinology*, **28**, 619.
Means (1937). The thyroid and its diseases. Philadelphia, Montreal, London.
Ross & Korenchevsky (1941). *J. Path. Bact.* **52**, 349.
Selye (1939). *J. Urol.* **42**, 637.
—— (1940). *Canad. med. Ass. J.* **42**, 113.
Smith & Perman (1940). *Endocrinology*, **27**, 110.
Sternheimer (1939). *Endocrinology*, **25**, 899.
Uotila (1940). *Endocrinology*, **26**, 129.

78. SERUM GONADOTROPHIN

I. PREPARATION OF A STABLE CONCENTRATE FROM PREGNANT MARE'S SERUM

By CLAUDE RIMINGTON AND IDWAL WYN ROWLANDS
From the National Institute for Medical Research, London, N.W. 3

(*Received 31 May 1941*)

SEVERAL different methods have been proposed for the preparation of gonadotrophic concentrates from pregnant mares' serum, but the majority of these fail to achieve adequate concentration of activity in relation to total weight, without serious loss of active material. Goss & Cole [1931] employed sodium sulphate fractionation and reported a ten-fold concentration of activity in the pseudoglobulin fraction. Evans *et al.* [1933] proposed using aluminium hydroxide as an adsorbing agent and this was applied to fresh plasma by Gustus *et al.* [1936]. Catchpole & Lyons [1934] used acetone for fractionation but achieved little separation from the serum proteins, while Meyer's [1935] method, using butanol and acetone, proved troublesome owing to the interference of inorganic salts.

The method which has so far proved most acceptable is that of Cartland & Nelson [1937] with the modifications introduced by Goss & Cole [1940]. It depends upon fractional precipitation of the serum with acetone at different *p*H levels; very large volumes of solvent are required. Cartland & Nelson state that a product substantially free from serum proteins may be obtained, representing 65 % of the initial activity. Goss & Cole find that recovery after the first stage, even when improved according to their directions, does not average more than 60 %, and the overall yields of their final preparations are not stated, although high potencies are claimed.

The use of salicylsulphonic acid at *p*H 1·9–2·2 to remove the inert proteins from active sera has been recommended by Rinderknecht *et al.* [1939]. One would expect such strongly acid solutions to cause not only a decrease in activity but also some irreversible splitting of the serum proteins, and in our hands both these expectations have been realized. Severinghaus *et al.* [1938] have obtained promising preliminary results with the ultracentrifuge applied to pregnant mare's serum.

A study of the foregoing publications shows that a method is still awaited which will achieve an initial concentration of the gonadotropic hormone, providing, without serious loss, a stable, solid substance of moderate activity but sufficiently refined to be easily soluble and suitable for clinical use. Such a concentrate can be obtained by the method described below in a yield representing on the average 73 % of the initial activity of the serum. The method is economical and easily operated on a large scale. It is proposed to use the concentrate so obtained for studies in further purification, and considerable progress has already been made in this direction.

Methods

Assays. All activities have been determined, in comparison with the International Standard, by the method described by Day & Rowlands [1940], using ten rats for each assay, and the results are expressed in International Units (I.U.).

Protein determinations have been calculated from the total nitrogen (micro-Kjeldahl) or determined by the colorimetric method of Greenberg [1929] using Folin's phenol reagent, a tyrosine standard and an empirically determined factor of 22·8 [see Rimington & van den Ende, 1940].

Bound carbohydrate has been determined by the orcinol method as described by Rimington [1940].

p*H values* have been determined electrometrically using the quinhydrone electrode or, where insufficient material was available, by indicators, using buffer solutions for comparison.

EXPERIMENTAL

Eight pregnant mares were available for this work and from each, after preliminary tests to confirm pregnancy, about six bleedings were made (see Table 3), each of approximately 2 l. of blood, the intervals between successive bleedings being a week and being so arranged as to cover the period during which gonadotrophic activity of the serum rises to its maximum [Day & Rowlands, 1940]. Although mating was not observed, the mares and stallion being pastured together, the estimated dates, based upon Day's [1940] method of rectal palpation for the diagnosis of pregnancy, are accurate to ±5 days. The blood was drawn into sterile receptacles containing 22 ml. of 10 % potassium oxalate per 2 l. of blood, the cellular elements were removed on the centrifuge and the resulting plasma clotted by addition, with vigorous agitation, of 3 ml. of 4 % calcium chloride to each 100 ml. of plasma. After straining, the serum was passed through a Seitz filter.

For kindly performing all these operations we are greatly indebted to Mr F. T. Day of the School of Agriculture, Cambridge.

After a small sample of each serum had been removed for biological assay, the remainder was stored at −10° until required for chemical work.

Exploratory experiments with ammonium sulphate

The possibility of fractionation by means of ammonium sulphate was investigated by separating from 100 ml. of serum, diluted with an equal volume of water, the globulin fraction at 50 % sat. (further differentiated by dialysis into eu- and pseudo-globulin), crystalbumin, albumin I (50–66 % sat.), albumin II (66–75 % sat.) and albumin III (75–100 % sat.). Each fraction was dialysed and the protein, bound carbohydrate and gonadotrophic activity determined with the results shown for a typical experiment in Table 1.

Table 1. *Fractionation of active serum by* $(NH_4)_2SO_4$

	Activity		
Fraction	% of total	I.U./mg. protein	Carbohydrate Total N
Whole serum	100	1·58	0·212
Euglobulin	7·5	2·56	0·029
Pseudoglobulin	45	1·14	0·030
Crystalbumin	18	1·80	0·100
Albumin I	33·5	6·08	0·329
„ II	0	0	0·538
„ III	0	0	1·370

There is no sharp separation of activity in any one fraction. Albumin I possesses the greatest potency, but this fraction represents, in all, only one-third of the total activity. Precipitation of the active material is complete at 66 % sat. with $(NH_4)_2SO_4$, a fact confirmed in experiments upon purer material, although the albumins II and III are relatively more rich in bound carbohydrate.

Acidic protein precipitants

A sharp separation of gonadotrophic activity from inert material follows the use of salicylsulphonic acid, trichloroacetic acid or metaphosphoric acid as a deproteinizing agent. Rinderknecht *et al.* [1939] recommended adding a 33·3 % solution of salicylsulphonic acid to the serum until the *p*H lay between 1·9 and 2·2, and claimed a recovery of nearly 100 % of the activity in the filtrate. This is surprising in view of the known instability of serum gonadotrophin in acid solution, and in our hands yields of less than 60 % were obtained. We found, moreover, that under such drastic conditions some disintegration of the serum protein occurred, and the precipitate was consequently difficult to centrifuge, while the supernatant liquids exhibited a milk-like opalescence.

Trichloroacetic acid produced fairly complete deproteinization, but a high concentration of the reagent was required, with a correspondingly high acidity and rapid destruction of activity. A large proportion of the activity was found, further, to be carried down with the precipitate.

An ideal protein precipitant for the present purpose should operate in low concentration and without producing a high resultant acidity; it should also, if possible, combine with the protein so that any free excess is reduced to the minimum. These conditions are fulfilled by metaphosphoric acid, which we consider to be a reagent worthy of much more attention for the purpose of deproteinizing tissue fluids or extracts, as an initial stage in the isolation of comparatively labile chemical substances.

The protein-metaphosphoric acid reaction has been studied recently by Perlmann & Herrmann [1938] and by Briggs [1940]. The former authors pointed out that a salt-like combination occurs between the reactants and considered that the PO_3 bound reached a maximum when the $PO_3 : NH_2$ ratio was 1; the protein is not denatured by the reagent. Briggs confirms the statement that metaphosphoric acid reacts with the basic groups of proteins to form salt-like combinations with very low dissociation tendency, but he finds the relationship between the quantity added, as Na metaphosphate, to the system and that bound by the protein to follow a Freundlich adsorption isotherm, so that the ratio $PO_3 : NH_2$ in the flocculate increases continuously with increase in metaphosphate. No similar reaction occurs between metaphosphate and amino-acids or other substances of low molecular weight, containing single basic groups.

Optimal conditions for the HPO_3 precipitation

The relations between the quantity of metaphosphoric acid added, the proportion of protein precipitated, the final *p*H of the solution, and the recovery of activity in precipitate and supernatant respectively, were explored as follows. To 10 ml. lots of an active serum diluted 1 : 3 with distilled water were added differing quantities of a freshly prepared $0{\cdot}3N$ HPO_3 solution. We have used throughout this work 'metaphosphoric acid, glacial in sticks' of British Drug Houses Ltd, and have found it convenient to work with the concentrations indicated. The addition was made dropwise to the well-stirred, ice-cold liquid; the precipitates were collected on the centrifuge, redissolved in water with the help of dilute alkali and made up to 10 ml. The *p*H of the supernatant fluids was determined on portions electrometrically, and on the remainders of these, and the redissolved precipitates, biological assays and determinations of protein (by phenol reagent) were performed. The results are shown in Fig. 1. At *p*H 3·5 the proportion of the total protein which is precipitated is 84 % and at *p*H 3·2 it has risen to 93·6 % with a corresponding improvement in the potency (activity per mg.) of the unprecipitated active fraction; on the other hand, at reactions more acid than *p*H 3·5 loss of hormone from the filtrate becomes appreciable. While, therefore, separation of inert protein is not completed at *p*H 3·5, this end-point has been chosen as that giving the best all-round results for the reaction. The quantity of HPO_3 required to reach this end-point varies slightly, in accordance, no doubt, with the total protein content of the sera, but averages 475 ml. of the 0·3*N* solution per l. of serum. When working up large batches of material, the precipitated protein metaphosphate has been removed by passing the mixture through a Sharples super-centrifuge and the cake has been washed by resuspending it in 0·01*N* HPO_3, using about 350–400 ml./l. of original serum, and again separating in the super-centrifuge. The combined filtrate and washings are neutralized by adding 20 % NaOH drop by drop with vigorous mechanical stirring.

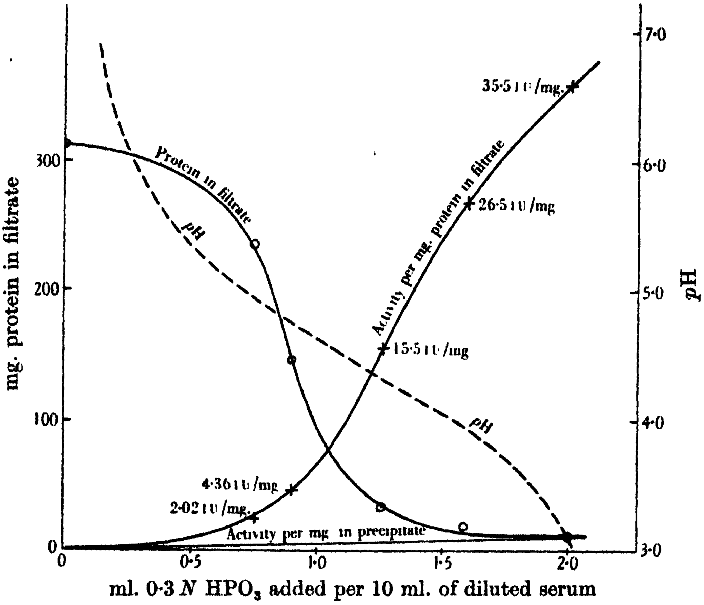


Fig. 1. Relations in the system: Pregnant mare's serum—metaphosphoric acid.

The average recovery at this stage has been 95 % of the original activity.

Adsorption of the active material upon benzoic acid

Katzman & Doisy [1934] were able to adsorb gonadotrophic hormone from pregnancy urine by adding to it a saturated solution of benzoic acid in acetone, whereupon benzoic acid was precipitated and could be filtered off, carrying a considerable proportion of the activity with it.

We endeavoured to achieve a similar adsorption from our metaphosphoric acid filtrates, but the yields were disappointing; not only was adsorption incomplete and variable, but there was also appreciable inactivation of gonadotrophic hormone. Benzoic acid suspensions have a *p*H of about 3, and we suspect that destruction is a result of this comparatively high acidity. Much better results were obtained by liberating benzoic acid in the solutions by adding a mineral acid to sodium benzoate. The system benzoic acid: sodium benzoate possesses buffering power, and a closer study showed that we could precipitate a sufficient amount of the free acid in a finely divided form in our solutions, both effectively and economically, without allowing the pH to fall below 4·5, a point at which there is little fear of inactivation of the hormone. Trials were made by using differing quantities of adsorbent at the same final pH, and also by varying the pH when the amount of benzoic acid in suspension was kept unchanged, from which it was found that adsorption takes place efficiently, from filtrates of widely varying activities, when the concentration of benzoic acid is about 5 % and the final pH is 4·5–5·0 but not greater than 5·5. These conditions are reached in practice by adding, to the filtrates, 60 g. sodium benzoate per l. (equivalent to 5 % benzoic acid) and then running, drop by drop, into the ice-cold, mechanically stirred solution, rather more than three-fourths of the equivalent quantity of $3N$ HCl. The final pH may be checked electrometrically or colorimetrically.

When working with large batches, the resulting precipitate has been collected by passing the mixture through a Sharples super-centrifuge, and unprecipitated benzoic acid has been recovered from the supernatant fluid, for purification and re-use, by addition of an excess of mineral acid.

We have omitted any aqueous washing of the benzoic acid with its adsorbed active material. The precipitate was scraped out and stirred with a quantity of acetone approximately equal to the volume of serum originally employed. This dissolves the benzoic acid and leaves the adsorbate as a finely flocculent material, which can be collected on the centrifuge, washed free from benzoic acid and moisture with three successive quantities of anhydrous acetone and one of dry ether, and then dried *in vacuo.*

Properties of 'initial powder'

The material obtained at this stage is a snow-white, light powder which dissolves slowly in water or buffer solution at pH 7·0–7·5, forming opalescent solutions which give positive biuret and Molisch tests, but contain no heat-coagulable protein. We have not been able to detect any change in the activity of samples kept during 8 months in stoppered bottles at room temperature. The yield of this material is about 6 g./l. of serum, and the activity ranges from 10 to 50 I.U./mg. It will be seen that only two simple chemical operations are necessary to obtain this comparatively stable product from active mares' serum which normally deteriorates unless stored under special conditions.

Factors affecting yields

From thirty-nine different sera, the activities of which varied over a wide range, we have been able to recover on the average 79 % of the original activity in the form of the stable initial powder. Details of the volumes, activities, yields etc. at each stage are presented in Table 2, and the mean data for each individual mare are summarized in Table 3. Our conclusions may be summarized as follows:

(1) *Volume of serum and weight of initial powder.* There is a fairly constant relationship between these quantities, irrespective of the activity of the serum employed, the mean yield being approximately 6 g./l. As will be seen later, we believe that this powder is made up, for the greater part, of some constituent, probably glycoprotein in nature (see p. 746), which carries the gonadotrophic hormone with it.

(2) *Activity of serum and activity of initial powder.* If the entrainment of gonadotrophically active substance by the inert material accompanying it in the initial powder were complete, or always in the same proportion, a straight-line relationship would be expected between the potencies of powder and serum.

Table 2. *Data on gonadotrophic activity and recovery up to stage of 'initial powder' from thirty-nine sera*

Mare and no. of sample	Stage of pregnancy days	Volume of serum ml.	Activity of serum I.U./ml.	HPO$_3$ filtrate Recovery of activity* %	Initial powder		
					Weight g.	Activity I.U./mg.	Recovery of activity* %
J 6	44	665	270	119	4·464	42	106
7	51	1375	390	86	†4·207 ‡4·647	40 38	65
8	58	1150	510	75	†3·677 ‡3·936	56 54	72
9	65	1135	450	105	8·838	40	91
10	72	1080	300	102	7·072	42	92
L 3	45	975	100	91	6·302	20	110
4	53	1190	90	113	7·084	14	93
5	58	1980	210	77	12·113	24	70
6	67	850	180	93	5·846	30	116
7	74	1590	180	75	10·738	20	87
8	81	1355	120	89	7·817	20	98
M 3	49	940	170	102	5·084	26	84
4	56	1065	280	91	5·442	28	51
5	63	1320	360	96	12·179	20	54
6	70	1080	360	80	6·235	30	48
7	77	960	290	114	6·251	32	74
8	84	1090	230	78	8·211	26	88
N 3	53	1055	150	100	5·152	30	98
4	60	1042	330	105	6·267	36	66
5	67	1430	390	84	†3·877 ‡4·227	57 52	79
6	74	1250	300	116	†3·941 ‡4·246	38 36	81
7	81	900	210	106	5·069	30	81
8	88	1275	72	90	8·788	9	87
P 3	45	1290	140	78	6·745	15	64
4	53	1070	220	100	6·758	28	82
5	58	2340	420	116	13·285	28	41
6	67	1240	450	111	9·236	24	40
7	74	1130	240	113	7·117	26	69
8	81	1215	255	85	6·876	29	65
Q 5	80	1040	100	74	6·632	7	44
5 A	80	620	64	—	3·230	11	90
R 2	59	1940	145	117	8·803	30	95
3	65	2170	155	106	12·348	24	88
S 4	47	817	145	101	3·484	34	102
5	54	1165	180	92	4·093	40	79
6	61	1390	240	94	7·683	34	78
7	68	1435	190	101	7·539	38	105
8	75	1220	145	92	6·324	18	65
9	82	1250	65	83	6·347	8	62

* Allowance has been made for samples withdrawn from the diluted serum for assay.

† 5% benzoic acid suspension. ‡ 10% benzoic acid suspension.

Fig. 2 demonstrates that this expectation is not fully realized. With sera containing up to 250 I.U./ml., the activity of the powder is very approximately a linear function of the activity of the serum, but with more highly active sera there is a progressive deviation from this ideal relationship. The experimental data can be more readily interpreted if the process of concentration of the activity be regarded as one of adsorption or, more strictly, of co-adsorption; gonadotrophin being first adsorbed by the inert serum constituent and this latter, with its attached active material, becoming secondarily adsorbed upon the benzoic acid precipitate liberated in the system. The latter adsorbent must be

Table 3. *Summary of data for individual mares*

					Initial powder		
No. of mare	No. of bleedings	Volume of serum extracted l.	Mean activity of serum I.U./ml.	Recovery in HPO_3 filtrate %	Total weight g.	Mean activity I.U./mg.	Recove %
J	5	5·405	385	97	36·841	45	85
L	6	7·940	147	90	49·900	21	96
M	6	6·455	282	94	43·400	27	67
N	6	6·952	242	100	41·585	36	82
P	6	8·285	287	101	50·020	25	60
Q	2	1·660	82	74	3·394	9	67
R	2	4·110	150	112	21·151	27	91
S	6	7·277	161	94	35·469	29	82
	39	48·084		95	281·760	27	79

present, however, in such quantity that adsorption upon it is virtually complete. The factor limiting the final yield of gonadotrophin would then be the relative quantity of the inert constituent supplied by the serum; increasing the amount of benzoic acid used should have little, if any, effect. This is borne out by the results of experiments in which the HPO_3 filtrates from highly active sera were divided, and one-half worked up as usual with 5 % of benzoic acid, the other half with twice this amount—see Table 4.

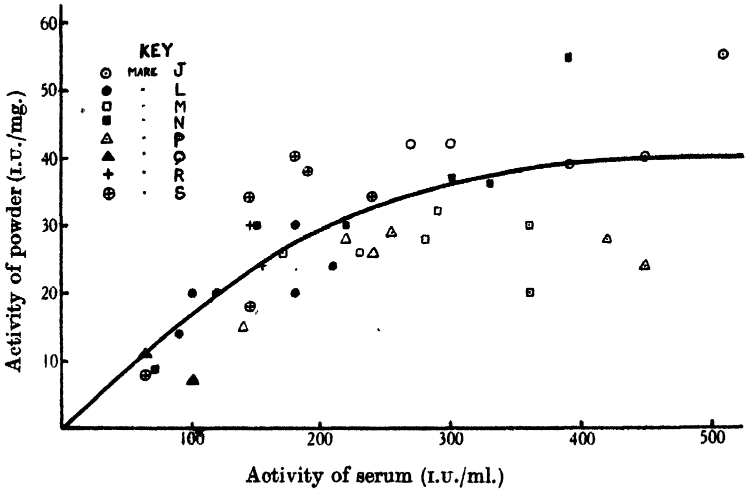


Fig. 2. Relation between activity of serum and potency of initial powder.

Table 4. *Effect of concentration of benzoic acid on recovery of gonadotrophin from highly active sera*

Serum	Activity I.U./ml.	Conc. of benzoic acid %	Wt. of powder g.	Activity I.U./mg.	Recovery of activity %
J 7	390	5	4·207	40	63
		10	4·647	38	66
J 8	510	5	3·677	56	70
		10	3·936	54	72
N 5	390	5	3·877	57	79
		10	4·227	52	79
N 6	300	5	3·941	38	80
		10	4·264	36	82

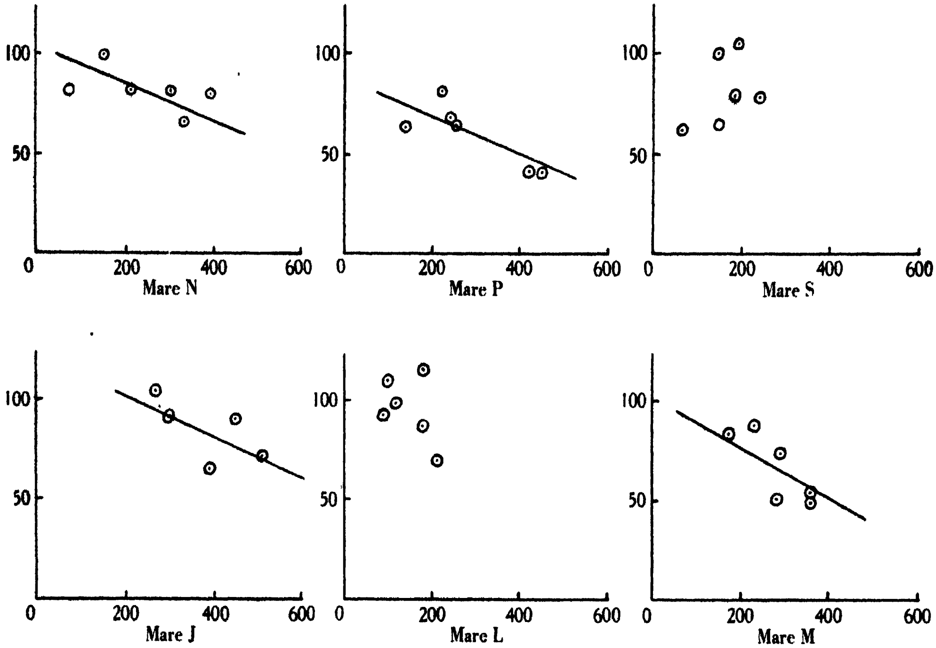


Fig. 3. Relation between activity of serum and percentage recovery in initial powder from individual mares. Abscissa = I.U./ml. of serum. Ordinate = percentage recovery.

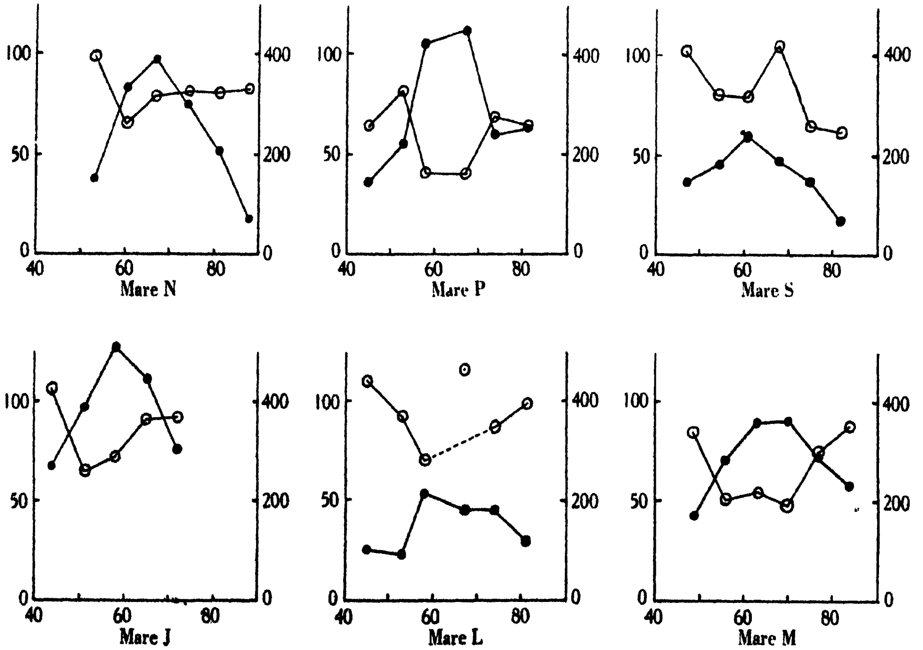


Fig. 4. Relation between activity of serum •—•—•, percentage recovery in initial powder o—o—o and stage of pregnancy for individual mares. Abscissa = stage of pregnancy in days. Ordinates = left: percentage recovery; right: I.U./ml. of serum.

(3) *Activity of serum and percentage recovery in initial powder.* Fig. 3 shows for each mare separately (omitting Q and R from which two bleedings only were obtained) the relationship between the original activity of the serum samples and the percentage of this activity finally recovered in the form of the 'initial powder'. In four out of six mares there is a regular fall in percentage recovery as the activity of the serum increases; in the remaining two cases, mares S and L, the peak activity usually encountered about the 65th day of pregnancy was much less marked, and the bleedings, therefore, provide insufficient evidence for such a comparison. These relationships are also brought out in Fig. 4, which relates stage of pregnancy to activity per ml. of serum and percentage recovery of activity.

The choice of a highly active serum as starting material for the preparation of gonadotrophic concentrate is not without its drawbacks. By pooling sera of higher and lesser activities, the optimal conditions for obtaining maximum recovery are likely to be more nearly approached.

A second feature discoverable from Fig. 3 is the difference between individual mares, some yielding sera from which a consistently better percentage yield of activity was obtained than others, e.g. the mean yield from the five bleedings of mare J was 85 % and that from the six bleedings of mare P only 60 % of the original activity of the sera. Such individual differences may, possibly, be due to differences in the proportion of 'inert protein adsorbent' in the sera.

Further concentration by fractional alcohol precipitation

The crude initial powder resulting from the benzoic acid adsorption can be further purified by alcohol precipitation at suitable *p*H levels; in particular, the sparingly soluble impurities can be eliminated and a material obtained assaying up to 625 I.U./mg. At this stage of the process we have been guided by the experience of Gurin *et al.* [1939] with urinary gonadotrophin, but we have modified their technique by adding alcohol to the neutral solution and then titrating with alcoholic acetic acid to *p*H 4·8, instead of shaking the powder directly with buffered alcohol. By this simplification, one complete set of manipulations, the extraction with 50 % alcohol at *p*H 6·0, is eliminated; moreover, by titrating instead of washing repeatedly with solvent a great saving of time is effected and, in our experience, higher yields are obtained. The method may be described as follows.

The initial powder is brought into solution by grinding it with forty times its weight of water containing 4·4 ml. 0·1 *N* NaOH per g. of powder, which is sufficient to bring the *p*H to about 7·4. It is advisable to stir the resulting mixture mechanically for some hours and to leave it standing in the ice chest overnight. An equal volume of alcohol ('Methcol') is then added and, while stirring mechanically, a 5 % solution of acetic acid in 50 % alcohol is added drop by drop until *p*H 4·8 is reached;[1] this requires approximately 1·1 ml./g. of initial powder.

The heavy precipitate which is formed is collected on the centrifuge after chilling the mixture for 1–2 hr., and washed three times by stirring with 50 % alcohol buffered at *p*H 4·8 (60 ml. *N* CH_3COONa + 40 ml. *N* acetic acid + 100 ml. water + 200 ml. alcohol) and recentrifuging. To the combined supernatant fluids an equal volume of alcohol is added (to 75 %), whereupon a fine flocculent precipitate is formed. The mixture is left overnight at 0° and the precipitate then collected on the centrifuge, washed once with each of the following: 80 % alcohol, absolute alcohol, acetone and dry ether; it is finally dried *in vacuo*.

---

[1] A small drop of the solution is mixed with a drop of water on a glazed plate; bromocresol green is added and the colour compared with that given by a similarly diluted drop of 50 % alcohol containing acetate buffer at *p*H 4·8. Values so obtained have been checked by the quinhydrone electrode.

We have found the yields by our method to be very good, approximately 92 % by weight of the initial powder being removed, leaving, on the average, 92 % of the activity in the concentrate (see Table 5).

Table 5. *Purification of initial powder by 50 °/₀ alcohol at* p*H 4·8*

	Initial powder		Concentrate		
No.	Wt. g.	Activity I.U./mg.	Wt. g.	Activity I.U./mg.	Recovery of activity %
L 5 (8)	0·189	36·4	0·0446	148	95·9
N 8 (1)	7·18	10	0·362	190	94·4
N 7 (1)	5·06	30	0·414	320	87·3
PMS 33 (1)	19·36	50	1·639	625	105·1
PMS 30	7·83	26	0·619	311	94·5
PMS 32	14·35	33·5	1·272	287	76·1
					Mean 92·2

During large-scale operations, a further economy might possibly be introduced by reworking the alcohol-insoluble residues for the modicum of activity they retain. As an example, the fraction of PMS 33 (1) insoluble in 50 % alcohol (see Table 5) and containing 5·3 I.U./mg. was redissolved in dilute alkali and the process of alcohol fractionation repeated. This material, which we designated PMS 33 (2), thus yielded a fraction soluble in 50 % alcohol, representing 81·3 % of the total activity and having a potency of 227 I.U./mg., while the residue assayed only 1·4 I.U./mg.

Properties of the product obtained after alcohol precipitation

The material obtained from the fraction soluble in 50 % alcohol at *p*H 4·8, but insoluble at 75 % concentration, is a white powder, very readily and completely soluble in water; solutions give positive biuret and glyoxylic tests. The gonadotrophic activity of our preparations has ranged from 150 to 625 I.U./mg., and there appears to be a correlation between the activities of the concentrates and those of the initial powders from which they were derived (see Table 5). The histological response elicited in the ovaries of hypophysectomized rats by the injection of the purified product is similar to that given by untreated serum.

Carbohydrate content of fractions prepared by 50 °/₀ alcohol

Determinations of carbohydrate content have been made at each of the principal stages in the concentration of serum gonadotrophin, but until the stage of alcohol fractionation was reached no reliable information could be gained concerning the nature of the hormone, owing to the large proportion of impurity still present. It was noted, however, that the initial powders afforded the relatively high figure of 7–8 % of bound hexose, resembling, in this respect, seromucoid and other typical glycoproteins.

The carbohydrate contents have now been determined of the 50 % alcohol concentrates in five out of the six samples listed in Table 5, and also in their corresponding fractions insoluble in 50 % alcohol at *p*H 4·8. The results show a striking difference between the two materials, the relatively inert fractions containing about 8 % of bound hexose and the active fractions from 2·5 to 3·5 % (see Table 6).

Table 6. *Carbohydrate content of fractions separated by 50 % alcohol at* p*H* *4·8*

	Insoluble fraction		Soluble fraction	
No.	Activity I.U./mg.	Hexose %	Activity I.U./mg.	Hexose %
N 8 (1)	0·7	8·32	190	2·94
N 7 (1)	2·7	8·42	320	2·54
PMS 33 (1)	5·3	7·40	625	3·44
PMS 33 (2)	1·4	7·80	227	2·41
PMS 30	1·9	8·38	311	2·84
PMS 32	1·9	7·78	287	3·87

This result is somewhat surprising, in view of the suggestion of Li *et al.* [1940] that their highly purified gonadotrophic extract from pregnant mare's serum is a glycoprotein, and the figure of 14·1 % hexose quoted in respect of it by them. Their statement that 'the carbohydrate determination may be taken as a measure of PMS potency in the purification of the hormone' is clearly invalid. Our preparations of about 600 I.U./mg. undoubtedly still contain much impurity, but carbohydrate content and gonadotrophic activity are, at this stage, running in opposite directions. An interesting illustration is afforded by sample PMS 33 (2). This was the alcohol-insoluble fraction of the large batch PMS 33 (1), and was refractionated by the same process (see above). The active fraction obtained (2 % of the weight) contained 81·3 % of the activity and 2·4 % hexose, while the insoluble fraction, containing 23·1 % activity, had a hexose content of 7·8 %, slightly higher than the initial figure of 7·4 % before fractionation.

DISCUSSION

The method most usually adopted for the preparation of active concentrates from pregnant mare's serum is that of Cartland & Nelson as modified by Goss & Cole [1940]. The latter authors state, however, that the yield from the initial acetone precipitation is less than 60 % of the original activity of the serum, and for their final products no yields are quoted. The method employs large volumes of acetone which would be difficult to recover, since they contain the water, salts etc. of the serum, and large vessels are required to handle the big bulk of fluids.

The method described in the present paper is simple and comparatively economical; the acetone and benzoic acid used can be recovered with ease, thus lessening working costs. To work up 100 l. of serum we calculate the requirements of the two methods as follows:

Cartland and Nelson's method	*Method here described*	
500 l. acetone (not easily recoverable)	1·4 kg. metaphosphoric acid	
Smaller quantities of alcohol, acids, etc.	20 kg. benzoic acid	Recoverable for re-use
	Approx. 100 l. acetone	
	Smaller quantities of alcohol, acids, etc.	

By our method, a material is obtained representing 73 % of the original activity of the serum and with an activity of 150–625 I.U./mg. For therapeutic use it may be mixed with a suitable proportion of an inert diluent.

Investigation is proceeding upon the further purification of the hormone with a view to its ultimate isolation and chemical characterization.

Protocol

As an example of the working of the method, a single preparation will be described in detail.

Serum N 7 from mare N when 81 days in foal; volume 900 ml.; activity assayed at time of preparation 210 I.U./ml.

To 900 ml. of serum were added 1800 ml. of water, and 20 ml. were removed for assay. The remainder was surrounded by ice and mechanically stirred while 430 ml. of $0 \cdot 3 N$ HPO_3 were added drop by drop at the rate of about 1 drop/sec. The *p*H, determined electrometrically, was then 3·3. The mixture was passed through a Sharples super-centrifuge and the cake of protein metaphosphate resuspended by grinding in 1 l. of $0 \cdot 01 N$ HPO_3. This mixture was passed through the machine and the clear fluid added to the main filtrate, which was neutralized to *p*H 7·0 by addition, with mechanical stirring, of sufficient 20% NaOH. The volume of filtrate was 4140 ml. 20 ml. were removed for assay. A solution of sodium benzoate was prepared by boiling 207 g. benzoic acid in 200 ml. water and 136 ml. of 50% NaOH, and cooling the mixture to room temperature.

The HPO_3 filtrate was surrounded by ice and mechanically stirred; the sodium benzoate solution was added and then $3 N$ HCl was allowed to drop into the briskly stirred mixture at a rate not greater than 1 drop per sec. 450 ml. of acid were required to bring the *p*H to 4·55. The mixture was put through the Sharples super-centrifuge and the cake of benzoic acid scraped out and stirred with about 1 l. of acetone.

When all the lumps had disintegrated, the fine precipitate was allowed to settle and the bulk of the supernatant acetone removed. The precipitate from the remainder was then collected on the centrifuge (using 200 ml. capacity glass bottles), washed three times on the centrifuge with about 400 ml. in all of anhydrous acetone and finally once with about 150 ml. of anhydrous ether. The last traces of ether were removed from the 'initial powder' by exposing it over sulphuric acid in a vacuum desiccator overnight.

Weight of powder recovered 5·069 g.
Activity 30 I.U./mg.
Recovery of original activity of serum 81%.

5·06 g. of initial powder were ground up with 200 ml. of water containing 2·2 ml. of *N* NaOH and the opalescent solution which had *p*H 7·4, was stirred mechanically for 2 hr. and then left in the ice chest overnight. 200 ml. of alcohol were added and then dropwise, while stirring, 7 ml. of 5% acetic acid in 50% alcohol, which brought the *p*H to 4·7. After chilling for 1·5 hr. the mixture was centrifuged and the precipitate washed on the centrifuge three times with 50% alcohol at *p*H 4·8 (see p. 744). To the combined supernatant fluids an equal volume of alcohol was added and the mixture left at 0° overnight. The small precipitate was collected on the centrifuge, washed once with 80% alcohol, absolute alcohol, acetone and dry ether, then freed from solvent *in vacuo* over sulphuric acid.

Weight of powder recovered 0·4141 g.
Activity 320 I.U./mg.
Recovery of activity 87·3%.
Overall recovery from serum 71%.

The material insoluble in 50% alcohol at *p*H 4·8 was dehydrated with acetone and ether. It weighed 4·475 g. and contained 2·7 I.U./mg., accounting for 8% of the total activity.

SUMMARY

A method is described for the preparation from the serum of pregnant mares of a stable, dry concentrate. The method is economical and is suitable for large-scale operation and depends firstly upon precipitation of inert serum proteins by metaphosphoric acid. Very little loss in gonadotrophic activity occurs at this stage. The active principle is then adsorbed upon benzoic acid and by solution of the latter in acetone a stable initial powder is obtained averaging 27 I.U./mg.

and accounting for 79 % of the activity (data compiled from thirty-nine individual preparations totalling 48 l. of serum).

The initial powder is further fractionated by means of titration in 50 % alcohol to *p*H 4·8; an insoluble portion representing 92 % by weight and practically devoid of activity is rejected, while from the solution the active fraction is recovered by further addition of alcohol. This material contains 150–625 I.U./mg. and is readily soluble in water. The average yield at this stage is 92 %, making an overall yield of 73 % of activity from the original serum.

The chemical properties of the active material have been investigated: it contains 2·5–3·5 % of hexose (orcinol method), while the inert fraction insoluble in 50 % alcohol at *p*H 4·8 contains 7·5–8·5 % of hexose.

Investigation is proceeding upon the further purification of the hormone.

We wish to thank Mr A. W. Hemmings for skilful technical assistance rendered during the course of this work.

REFERENCES

Briggs (1940). *J. biol. Chem.* **134**, 261.
Cartland & Nelson (1937). *J. biol. Chem.* **119**, 59.
Catchpole & Lyons (1934). *Amer. J. Anat.* **55**, 167.
Day (1940). *J. agric. Sci.* **30**, 244.
—— & Rowlands (1940). *J. Endocrinol.* **2**, 255.
Evans, Gustus & Simpson (1933). *J. Exp. Med.* **58**, 569.
Goss & Cole (1931). *Endocrinology*, **15**, 214.
—— —— (1940). *Endocrinology*, **26**, 244.
Greenberg (1929). *J. biol. Chem.* **82**, 545.
Gurin, Bachman & Wilson (1939). *J. biol. Chem.* **128**, 525.
Gustus, Meyer & Woods (1936). *J. biol. Chem.* **114**, 59.
Katzman & Doisy (1934). *J. biol. Chem.* **106**, 125.
Li, Evans & Wonder (1940). *J. gen. Physiol.* **23**, 733.
Meyer (1935). *Proc. Soc. exp. Biol., N.Y.*, **33**, 433.
Perlmann & Herrmann (1938). *Biochem. J.* **32**, 926.
Rimington (1940). *Biochem. J.* **34**, 931.
—— & van den Ende (1940). *Biochem. J.* **34**, 941.
Rinderknecht, Noble & Williams (1939). *Biochem. J.* **33**, 381.
Severinghaus, Levin & Chiles (1938). *Endocrinology*, **23**, 285.

I. A. R. I. 75.

IMPERIAL AGRICULTURAL RESEARCH
INSTITUTE LIBRARY
NEW DELHI.

Date of issue.	Date of issue.	Date o issue.
26.8.67		